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THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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VOLUME LXXV
AUGUST TO DECEMBER, 1938

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

The Biological Bulletin is issued six times a year. Single numbers, $1.75. Subscription per volume (3 numbers), $4.50.

Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Mass., between June 1 and October 1 and to the Institute of Biology, Divinity Avenue, Cambridge, Mass., during the remainder of the year.

4.58

Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

LANCASTER PRESS, INC., LANCASTER, PA.

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THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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Fiftieth Year

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II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

LIBRARY

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, Henry B. Pierce, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 11.30 A.M., daylight saving time, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years, and in addition there shall be two groups of Trustees as follows: (*a*) Trustees *ex officio,* who shall be the President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (*b*) Trustees Emeritus, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next annual meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee Emeritus for life. The Trustees *ex officio* and Emeritus shall have all rights of the Trustees except that Trustees Emeritus shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. Inasmuch as the time and place of the Annual Meeting of Members is fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of said meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen: Herewith is my report as Treasurer of the Marine Biological Laboratory for the year 1937.

The accounts have been audited by Messrs. Seamans, Stetson and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and is open to inspection by members of the Corporation.

At the end of the year 1937, the book value of the Endowment Funds in the hands of the Central Hanover Bank and Trust Company as Trustee, was

General Fund,	Securities	$ 915,302.04
	Real Estate	9,595.71
	Cash	867.87
Library Fund,	Securities	174,141.23
	Real Estate	20,000.00
	Cash	102.85
		$1,120,009.70

The income collected from these Funds was as follows:

General Endowment	$39,945.92
Library Fund	7,345.93
	$47,291.85

Of this $4,899.39 represented the collection of arrears of interest outstanding at the beginning of 1937, reducing this item to

Arrears *General Fund*	$10,105.86
Arrears *Library Fund*	2,650.00
	$12,755.86

The dividends from the General Biological Supply House amounted to $10,160.

Retirement Fund. A total of $4,060 was paid in pensions. The Fund at the end of the year consisted of securities of the book value

of	$15,769.01	
Real Estate	2,260.00	
Cash	212.82	$18,241.83

The interest in arrears has been reduced to less than $5.00.

Plant Assets. The land (exclusive of Gansett and Devil's Lane), buildings, equipment and library, represented an investment of

	$1,777,408.38	
less reserve for depreciation	495,772.66	$1,281,635.72

Income and Expenses. Expenses including $24,343.77 depreciation exceeded income by $4,370.22.

There was expended from current funds net $19,549.01 for plant account.

After careful consideration by the Executive Committee and the accountants the rate of depreciation was changed as follows:

	Old Rate per annum	*New Rate per annum*
Buildings		
Brick	2%	1 %
Wood, etc.	2%	2 %
Equipment	5%	5 %
Library		
Back sets	5%	none
Other	5%	2½%

At the end of the year the Laboratory owed on mortgage $8,500, notes payable $10,500, and accounts payable $5,947.12. It had accounts and notes receivable of $13,562.36 and $12,298.54 in cash and bank accounts in its current assets.

Following is the balance sheet, the condensed statement of income and outgo, and the surplus account all as set out by the accountants:

EXHIBIT A

Marine Biological Laboratory Balance Sheet, December 31, 1937

Assets

Endowment Assets and Equities:		
Securities and Cash in Hands of Central Hanover Bank and Trust Company, New York, Trustee—Schedules I-a and I-b	$1,120,009.70	
Securities and Cash—Minor Funds—Schedule II	8,513.51	$1,128,523.21

Plant Assets:			
Land—Schedule IV	$ 110,884.58		
Buildings—Schedule IV	1,238,924.01		
Equipment—Schedule IV	162,833.58		
Library—Schedule IV	264,766.21	$1,777,408.38	
Less Reserve for Depreciation		495,772.66	
		$1,281,635.72	
Cash in Dormitory Building Fund		223.24	
Cash in Reserve Fund		24.65	$1,281,883.61
Current Assets:			
Cash		$ 12,298.54	
Accounts and Notes—Receivable		13,562.35	
Inventories:			
Supply Department	$ 38,282.75		
Biological Bulletin	12,764.61	51,047.36	
Investments:			
Devil's Lane Property	$ 43,916.11		
Gansett Property	5,718.49		
Stock in General Biological Supply House, Inc.	12,700.00		
Securities and Cash—Retirement Fund—Schedule V	18,241.83	80,576.43	
Prepaid Insurance		3,657.61	
Items in Suspense (Net)		71.65	$ 161,213.94
			$2,571,620.76

Liabilities

Endowment Funds:			
Endowment Funds—Schedule III	$1,119,594.24		
Reserve for Amortization of Bond Premiums	415.46	$1,120,009.70	
Minor Funds—Schedule III		8,513.51	$1,128.523.21
Plant Liabilities and Funds:			
Mortgage—Payable, Howes Property		$ 8,500.00	
Notes—Payable a/c Bar Neck Property Purchase		8,500.00	
Donations and Gifts—Schedule III		1,034,699.61	
Other Investments in Plant from Gifts and Current Funds		230,184.00	$1,281,883.61
Current Liabilities and Surplus:			
Accounts—Payable		$ 5,947.12	
Notes—Payable		2,000.00	
		$ 7,947.12	
Current Surplus—Exhibit C		153,266.82	$ 161,213.94
			$2,571,620.76

EXHIBIT B

Marine Biological Laboratory Income and Expense, Year Ended December 31, 1937

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund ...		$ 39,945.92		$ 39,945.92
Library Fund		7,345.93		7,345.93
Donations		36.68		36.68
Instruction	$ 8,219.29	10,005.00		1,785.71
Research	4,281.68	16,695.00		12,413.32
Evening Lectures	33.50		$ 33.50	
Biological Bulletin and Membership Dues	9,282.60	9,712.40		429.80
Supply Department—Schedule VI	43,869.93	39,640.88	4,229.05	
Mess—Schedule VII	28,051.56	28,647.58		596.02
Dormitories—Schedule VIII ..	25,813.00	13,264.18	12,548.82	
(Interest and Depreciation charged to above three Departments—See Schedules VI, VII, and VIII)	*23,698.07*			23,698.07
Dividends, General Biological Supply House, Inc.		10,160.00		10,160.00
Rents:				
Bar Neck Property		3,973.95		3,973.95
Bay Shore Property	249.55	132.00	117.55	
Howes Property	701.26	480.00	221.26	
Janitor's House	18.85	360.00		341.15
Newman Cottage	142.30	250.00		107.70
Danchakoff Cottage	282.79	750.00		467.21
Sale of Duplicate Library Sets		153.04		153.04
Interest on Notes—Receivable .		240.00		240.00
Sundries		70.76		70.76
Maintenance of Plant:				
Buildings and Grounds	23,733.29		23,733.29	
Chemical and Special Apparatus Expense	14,355.36		14,355.36	
Library Expense	7,639.80		7,639.80	
Truck Expense	1,023.44		1,023.44	
Workmen's Compensation Insurance	472.30		472.30	
Sundry Expense	46.17		46.17	
General Expenses:				
Administration Expense	14,713.71		14,713.71	
Endowment Fund Trustee and Safe-keeping	1,001.95		1,001.95	
Interest on Notes and Mortgage—Payable	639.11		639.11	
Bad Debts	1,016.40		1,016.40	
Reserve for Depreciation	24,343.77		24,343.77	
	$186,233.54	$181,863.32	$106,135.48	$101,765.26

Excess of Expenses over Income carried to Current Surplus—Exhibit C	4,370.22	4,370.22
	$186,233.54	$106,135.48

EXHIBIT C

MARINE BIOLOGICAL LABORATORY, CURRENT SURPLUS ACCOUNT, YEAR ENDED DECEMBER 31, 1937

Balance, January 1, 1937			$156,677.06
Add:			
Reserve for Depreciation charged to Plant Funds			24,343.77
			$181,020.83
Deduct:			
Payments from Current Funds during Year for Plant Assets as shown in Schedule IV,			
Land		$ 1,135.19	
Buildings		361.17	
Equipment		3,823.32	
Library		14,388.83	
		$19,708.51	
Less Received for Plant Assets Disposed of		159.50	
		$19,549.01	
Pensions and Allowances Paid	$4,060.00		
Expenses on account of Retirement Fund Securities	540.38		
	$4,600.38		
Less Retirement Fund Income	765.60	3,834.78	
Excess of Expenses over Income as shown in Exhibit B		4,370.22	27,754.01
Balance, December 31, 1937—Exhibit A			$153,266.82

BIOLOGICAL LABORATORY WOODS HOLE LIBRARY

Respectfully submitted,

LAWRASON RIGGS, JR.,
Treasurer

V. THE REPORT OF THE LIBRARIAN

A report of the expenditure of the budget assigned in 1937 at the usual total of $18,800 is as follows: books, $163.58; serials, $5,229.29; binding, $1,430.77; express, $103.65; supplies, $402.71; salaries, $7,150.00; back sets, $4,304.81; credit for sale of duplicates, $153.04; total, $18,631.77. The amount contributed by the Woods Hole Oceanographic Institution was separately accounted, $638.36.

Of the total number 1,330 current serials entered by the Library in 1937, the Marine Biological Laboratory purchased 378, the Woods Hole Oceanographic Institution 40; exchanged using the "Biological Bulletin" 621, using the Woods Hole Oceanographic Institution publications 75; received as gifts 216, seven of the last as gifts to the Woods Hole Oceanographic Institution. The number of books purchased was 47, eight of these out of the Woods Hole Oceanographic Institution budget. Gifts of 70 books came during the year, 34 from various publishers, 13 from the authors to the Marine Biological Laboratory, 21 from various sources, the greatest number having been presented by Prof. Frank R. Lillie, and 2 to the Woods Hole Oceanographic Institution direct from the author. By purchase 24 back sets of serials were completed by the Marine Biological Laboratory, 1 by the Woods Hole Oceanographic Institution; 12 partially completed by the former and 1 by the latter. By exchange of duplicate serials 8 further back sets were partially filled in for the Library.

The new reprints filed in 1937 totalled 7,042; current issues of 1936 but received this year 685 and these added to 1936 receipts total 1,940; 765 reprints current of 1937; reprints of earlier date, 4,337. Professor Frank R. Lillie presented 1,261 of these; Prof. E. W. Gudger, 100; and several hundred were presented by Prof. G. C. Wheeler; the remainder came to us from authors who filled in complete or nearly complete sets of their early reprints for us.

A summary of the above gives the interesting figures for the Library holdings as 1,330 current serial publications, 2,705 current (1936–37) reprints; 90 current books; 25 back sets of serials completed; 4,337 old reprints and 10 old books received; total bound volumes now in the Library, 43,442; and total reprints 102,022.

VI. THE REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I beg to submit herewith a report of the fiftieth session of the Marine Biological Laboratory for the year 1937.

1. *Attendance.* Following an almost stationary registration of approximately 315 investigators during the depression years of 1932 to 1935 inclusive the attendance in 1936 showed a sharp increase, rising in that year to 359, which almost equalled the highest previous figure of 362 in 1931. During the year covered by this report a further increase has brought the number of investigators to 391, the largest in the entire history of the Laboratory. When to this number is added that of the students in the courses, which as for many years past has been about the

maximum that can be accommodated in the various class-rooms, the total attendance of 524 for 1937 is also seen to be the largest yet recorded, as is likewise the number of institutions represented (165 as compared with the next-highest figure of 158 in 1936).

While this rapid growth of the Laboratory, and in particular the widespread nature of its present scientific connections with American and foreign educational institutions, is most gratifying, it at the same time raises several serious problems. As has been pointed out in previous reports of the Director, neither the facilities of the Laboratory itself nor the living accommodations in the village of Woods Hole are such as to permit much further expansion; indeed, the registration for 1937 seems already to have passed the figure which experience has shown to be the optimum under existing conditions. Since there are several possible ways of meeting this situation, which must be faced without delay, and since the policies now adopted by the Laboratory are likely to affect profoundly the character of its future development, it has seemed wise to refer the question of attendance, as well as several others of importance, to the special Committee on Policy to which further reference will be made below.

The usual tabulation of the rise and fall of attendance by ten-day intervals for the past 10 years follows.

		1927	1928	1929	1930	1931	1932	1933	1934	1935	1936	1937
May	30.......	7	15	9	6	6	8	11	12	11	14	10
June	10.......	50	64	55	50	51	54	46	54	43	41	57
"	20.......	114	140	139	153	153	127	129	137	127	142	145
"	30.......	212	240	197	208	217	172	184	196	174	190	214
July	10.......	247	281	238	253	258	225	235	249	226	242	285
"	20.......	247	282	242	250	273	245	253	256	232	260	301
"	30.......	245	272	249	253	281	248	255	248	257	272	320
August	10.......	234	250	256	254	302	257	261	264	245	282	311
"	20.......	208	226	243	245	280	236	244	250	235	266	307
"	30.......	168	183	220	204	239	190	205	211	192	223	248
September	10.......	110	112	157	122	136	129	117	93	94	121	126
"	20.......	50	43	59	44	69	58	45	38	26	47	39
"	30.......	12	14	14	8	14	13	12	9	11	20	14

2. *The Report of the Treasurer.* A comparison of the corresponding figures from the reports of the Auditors for 1936 and 1937 shows for the latter year a decrease in income from endowment of approximately $2,900, the actual amounts received for the two years in question having been $50,192.56 and $47,291.85. When account is taken of the fact that of the total sum received in 1937 $4,899.39 represented the collection of arrears rather than regular interest, it is obvious that the Laboratory at present is attempting care for the needs of the largest

LIBRARY

number of investigators in its history with an income from endowment approximately $15,000 below its pre-depression level. That difficulties have been encountered in so doing is not surprising; but that these difficulties have so far been surmounted is due to the fine spirit of coöperation shown by all the investigators in adapting themselves to changed conditions. However, in view of the fact that the recent decline in the rates of interest obtainable from sound investments shows no indication of a reversal or even of a complete arrest, it is obvious that one of the most urgent needs of the Laboratory at present is for an increase in its endowment funds sufficient to bring its income from this source back at least to its pre-depression level.

Next in importance to its endowment as a source of income to the Laboratory are the subscriptions received from institutions and individuals for research space. The amount received from these sources in 1937 shows an encouraging increase of approximately $2,500 over the previous year and of more than $4,000 over the lowest level reached during the depression. It should be noted, however, that the sum received in 1937, while the highest in six years, still falls short by almost $1,500 of that received in 1931 when the attendance was appreciably smaller. In the early days of the Laboratory it was only through the support received from subscribing and coöperating institutions that its continued existence was possible. While in recent years the importance of this support may have seemed less obvious, it has in fact always been indispensable, and at no time more clearly so than at present.

Of the other usual sources of income, more favorable figures are shown by the Mess and the dormitories for 1937 than for several years past. This improvement is partly a real one due to an actual increase in income, resulting from the increased attendance and in particular from a lengthening of the most active season. In part, however, it is one of bookkeeping, due to the adoption of a new method for the estimation of depreciation which seemed to the Auditors and to the Executive Committee to be, on the whole, a fairer one than that previously used. This matter is discussed in considerable detail in a recent special report prepared by the Auditors, Messrs. Seamans, Stetson and Tuttle, which may be consulted for further information.

As for a number of years past, dividends received from the General Biological Supply House have been of material assistance in compensating for the decreases in sales by the Supply Department, which under the prevailing business conditions have been unavoidable.

3. *The Report of the Librarian.* During the year 1937 the growth of the library has continued at a rate which in view of the diminished income of the Laboratory from endowment funds may be considered to

be very satisfactory. While purchases of ordinary books and of back sets of journals were of necessity considerably curtailed in 1937, there was only a very slight decrease in the number of journals currently received. It is to the large number and convenient arrangement of these journals and their back sets that the library of the Marine Biological Laboratory owes its unique value to workers in Biology and related fields. Another extremely valuable feature of the library is its extensive collection of reprints which now exceeds 100,000, indexed and readily accessible to all workers at the Laboratory. The thanks of the Laboratory are due to those who in 1937, as well as in previous years, have generously contributed to this important collection. The following tabulation shows at a glance the character of the growth of the library during the past 10 years.

	1928	1929	1930	1931	1932	1933	1934	1935	1936	1937
Serials received currently......	874	985	1,060	1,080	1,126	1,137	1,197	1,271	1,339	1,330
Total number of bound volumes.	26,500	28,300	31,500	33,800	36,000	37,400	38,600	40,200	42,000	43,400
Reprints.........	51,000	59,000	64,000	70,000	76,000	81,000	86,000	92,000	95,000	102,000

4. *Changes in Personnel.* At the annual meeting of the Trustees held on August 10, 1937, the resignation as Director of Dr. M. H. Jacobs, who had become Associate Director of the Laboratory in 1925 and Director in 1926 and who now wished to be able to devote a larger proportion of his time to scientific research and writing, was received, and was accepted with expressions of regret. At the same time formal approval was given to the action of the President in having appointed several weeks previously a Special Committee to deal with the situation thus arising, of whose existence he and the Executive Committee had already been informed. Authority was given by the Board of Trustees to this Committee, which consisted of Drs. Calkins, Irving, Jacobs, Morgan, Parker, Stockard, and Conklin, Chairman, together with the Executive Committee, to take such steps as might be necessary to fill the vacant position. After several meetings of the Special Committee, in consultation with the Executive Committee, during the late summer of 1937 it had not proved feasible to arrive at a complete solution of the problem, but the important step had been taken of selecting for the position of Assistant Director, which had been vacant since 1925, Dr. Charles Packard of Columbia University, a man whose personal qualities and scientific attainments and long experience with the affairs of the Laboratory admirably fitted him to take over the administrative duties given up by the retiring Director on January 1, 1938.

5. *The Board of Trustees.* At the meeting of the Corporation held on Tuesday, August 11, Dr. T. H. Morgan, who as one of its most

BIOLOGICAL LABORA
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distinguished members and as a Trustee since 1897 has contributed much to the present scientific standing of the Marine Biological Laboratory, was elected Trustee Emeritus. At the same meeting Dr. W. C. Curtis of the University of Missouri was chosen to fill the place thus created in the Class of 1941.

It is a painful duty to record the loss by death in very close succession in the spring of 1937 of three highly respected members or former members of the Board of Trustees: Dr. Milton Jay Greenman, who died on April 7, in his seventy-first year, Dr. William Morton Wheeler, who died on April 19, in his seventy-third year and Dr. Elias Potter Lyon who died on May 4, in his seventieth year. Suitable memorials commemorating the scientific achievements, and in particular the services to the Marine Biological Laboratory, of these three distinguished biologists will be presented at the next meeting of the Board of Trustees.

6. *Committee on Policy.* Several very important questions, of which that of the rapidly increasing attendance and the need for larger endowment funds mentioned above are typical, have for some time seemed to demand for their proper solution the collective wisdom and experience of a considerable number of persons who by close personal association with the affairs of the Marine Biological Laboratory have become thoroughly acquainted with its peculiar problems. Accordingly, the Board of Trustees at its meeting on August 11, 1937 authorized the President to appoint a Committee on Policy, not to exceed nine members, to consider the present status of the Laboratory and its policy for the future and to report at the next annual meeting of the Trustees. The committee appointed by the President, consisting of Drs. Calkins, Curtis, Goodrich, Jacobs, Morgan, Parker, Redfield, Stockard and Conklin, Chairman, has already held several very profitable meetings, with further discussion of various matters by correspondence, and its final report is now in the course of preparation.

7. *Gifts.* During the summer of 1937, Mr. Charles R. Crane, to whom the Laboratory is already so deeply indebted for material assistance and personal interest on numerous occasions in the past, added another much appreciated gift in the form of a very handsome sundial, in a setting appropriately beautified by plantings of grass and shrubs, opposite the main Brick Building by the side of Great Harbor. The sundial was designed by the late Professor Ernest Yalden, to whom it is a permanent and very fitting memorial. At the dedication exercises held in the Auditorium of the Laboratory on the morning of August 23 after introductory remarks by Dr. Frank R. Lillie the speech of presentation was made on behalf of Mr. Crane by Dr. H. C. Bradley.

and the speech of acceptance on behalf of the Laboratory by Dr. E. G. Conklin.

8. *The Cedar Swamp.* For many years an important collecting ground for certain types of biological material used by students in the courses as well as by investigators at the Laboratory has been the large Cedar Swamp lying to the southeast of the village of Woods Hole between the Falmouth road and the railroad. Swamps of this type are highly characteristic of the Cape Cod region but, in their primitive condition at least, are each year becoming more rare because of the cutting of timber, drainage, etc. When, therefore, during the past year an opportunity arose to purchase the tract in question from the New York, New Haven and Hartford Railroad, advantage was taken of it without delay, in order to preserve for the future use of Woods Hole biologists this very interesting region. The tract in question has an area of about 10 acres of which approximately half an acre is occupied by a shallow lake. Its greatest usefulness to the Laboratory both as a collecting ground and a suitable locality for future ecological studies will probably be secured by keeping it with a minimum of disturbance in its present wild condition.

9. *Lectures and Scientific Meetings.* During the summer of 1937 the number of regular evening lectures was 14, and in addition at 9 less formal evening meetings 37 shorter papers were presented and discussed. Of unusual interest was the final scientific meeting on August 26th and 27th, which was devoted exclusively to reports on work accomplished during the current season. The number of papers submitted for this meeting was so large that three full half-days were required for their presentation, with an additional half-day for demonstrations. Both in the number and excellence of its papers the 1937 meeting surpassed any previously held. Abstracts of nearly all the papers presented or read by title will be found in the October 1937 issue of the Biological Bulletin. Together they fill 38 pages of small print, and furnish very concrete evidence that the recent increases in attendance have not run ahead of the scientific productiveness of the Laboratory.

In addition to its own scientific meetings the Marine Biological Laboratory also acted as host to the Genetics Society of America, when between August 30th and September 1st it held its fourth and in many respects its most successful Woods Hole summer meeting.

There are appended as parts of the report:

1. The Staff, 1937.
2. Investigators and Students, 1937.
3. A Tabular View of Attendance, 1933–1937.
4. Subscribing and Coöperating Institutions, 1937.

5. Evening Lectures, 1937.
6. Shorter Scientific Papers, 1937.
7. General Scientific Meeting, 1937.
8. Members of the Corporation, August, 1937.

Respectfully submitted,

M. H. JACOBS,
Director.

1. THE STAFF, 1937

Merkel H. Jacobs, *Director,* Professor of General Physiology, University of Pennsylvania.
Associate Director: ——

ZOÖLOGY

I. Investigation

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
E. G. Conklin, Professor of Zoölogy, Princeton University.
Caswell Grave, Professor of Zoölogy, Washington University.
H. S. Jennings, Professor of Zoölogy, Johns Hopkins University.
Frank R. Lillie, Professor of Embryology Emeritus, The University of Chicago.
C. E. McClung, Professor of Zoölogy, University of Pennsylvania.
S. O. Mast, Professor of Zoölogy, Johns Hopkins University.
T. H. Morgan, Director of the Biological Laboratory, California Institute of Technology.
G. H. Parker, Professor of Zoölogy Emeritus, Harvard University.
E. B. Wilson, Professor of Zoölogy Emeritus, Columbia University.
Lorande L. Woodruff, Professor of Protozoölogy, Yale University.

II. Instruction

T. H. Bissonnette, Professor of Biology, Trinity College.
P. S. Crowell, Jr., Instructor in Biology, Miami University.
C. E. Hadley, Associate Professor of Biology, New Jersey State Teachers College at Montclair.
F. R. Kille, Assistant Professor of Zoölogy, Swarthmore College.
S. A. Matthews, Associate in Anatomy, School of Medicine, University of Pennsylvania.
L. P. Sayles, Assistant Professor of Biology, College of the City of New York.
A. J. Waterman, Assistant Professor of Biology, Williams College

Junior Instructors

A. M. Lucas, Associate Professor of Zoölogy, Iowa State College.
J. S. Rankin, Teaching Fellow in Zoölogy, Amherst College.

PROTOZOÖLOGY

I. Investigation

(See Zoölogy)

II. Instruction

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
Elizabeth Drumtra Hughes, Instructor in Zoölogy, Wilson College.
G. W. Kidder, Instructor in Zoölogy, College of the City of New York.

EMBRYOLOGY

I. Investigation

(See Zoölogy)

II. Instruction

L. G. Barth, Assistant Professor of Zoölogy, Columbia University.
Hubert B. Goodrich, Professor of Biology, Wesleyan University.
Viktor Hamburger, Assistant Professor of Zoölogy, Washington University.
Charles Packard, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.
Oscar Schotté, Assistant Professor of Biology, Amherst College.

PHYSIOLOGY

I. Investigation

William R. Amberson, Professor of Physiology, University of Tennessee.
Harold C. Bradley, Professor of Physiological Chemistry, University of Wisconsin.
Walter E. Garrey, Professor of Physiology, Vanderbilt University Medical School.
Ralph S. Lillie, Professor of General Physiology, The University of Chicago.
Albert P. Mathews, Professor of Biochemistry, University of Cincinnati.

II. Instruction

Teaching Staff

Laurence Irving, Professor of Experimental Biology, University of Toronto.
Robert Chambers, Professor of Biology, New York University.
J. K. W. Ferguson, Assistant Professor of Physiology, Ohio State University.
Kenneth C. Fisher, Instructor in Zoölogy, University of Maine.
Rudolf Höber, Visiting Professor of Physiology, University of Pennsylvania.
C. Ladd Prosser, Assistant Professor of Physiology, Clark University.

Junior Instructors

F. J. M. Sichel, Instructor in Physiology, Howard University School of Medicine.

BOTANY

I. Investigation

C. E. Allen, Professor of Botany, University of Wisconsin.
S. C. Brooks, Professor of Zoölogy, University of California.
B. M. Duggar, Professor of Physiological and Economic Botany, University of Wisconsin.
Ivey F. Lewis, Professor of Biology, University of Virginia.
Wm. J. Robbins, Professor of Botany, University of Missouri.

II. Instruction

William Randolph Taylor, Professor of Botany, University of Michigan.
Francis Drouet, Research Fellow, Yale University.
G. W. Prescott, Assistant Professor of Biology, Albion College.

GENERAL OFFICE

F. M. MacNaught, Business Manager.
Polly L. Crowell, Assistant.
Edith Billings, Secretary.

RESEARCH SERVICE AND GENERAL MAINTENANCE

Samuel E. Pond, Technical Mgr.
O. W. Richards, Chemical Service.
G. Failla, X-ray Physicist.
T. E. Larkin, Superintendent.
W. C. Hemenway, Carpenter.
Lester F. Boss, Technician.
J. D. Graham, Glassblower.
J. T. Simonton, Assistant.
Elbert P. Little, X-ray.
R. T. Goffin, Technical Ass't.

LIBRARY

Priscilla B. Montgomery (Mrs. Thomas H. Montgomery, Jr.), Librarian.
Deborah Lawrence, Secretary.
Doris Endrejat, Mary A. Rohan, Assistants.

SUPPLY DEPARTMENT

James McInnis, Manager.
Milton B. Gray, Collector.
A. M. Hilton, Collector.
A. W. Leathers, Shipping Dept.
Geoffrey Lehy, Collector.
Walter Kahler, Collector.
Ruth S. Crowell, Secretary.
Anna N. Hall, Secretary.

THE GEORGE M. GRAY MUSEUM

George M. Gray, Curator Emeritus.

2. INVESTIGATORS AND STUDENTS, 1937

Independent Investigators

Abell, Richard G., Fellow in Anatomy, University of Pennsylvania, School of Medicine.

Abramowitz, A. A., Research Assistant, Harvard University.

Addison, William H. F., Professor of Normal Histology and Embryology, University of Pennsylvania.

Allee, W. C., Professor of Zoölogy, The University of Chicago.

Amberson, William R., Professor of Physiology, University of Tennessee, School of Medicine.

Anderson, R. L., Professor of Biology, Johnson C. Smith University.

Andrew, Warren, Teaching Fellow, University of Georgia, School of Medicine.

Appel, Frederick W., Associate Professor of Biology, St. John's College.

Armstrong, Philip B., Professor of Anatomy, University of Alabama Medical School.

Bailey, Percy L., Jr., Assistant Professor of Biology, College of the City of New York.

Ballard, W. W., Assistant Professor of Anatomy and Biology, Dartmouth College.

Bargeton, Daniel E., Assistant in Physiology at Faculty of Medicine, University of Paris.

Barth, L. G., Assistant Professor of Zoölogy, Columbia University.

Beadle, G. W., Assistant Professor, Harvard University.

Bear, Richard S., Research Assistant, Washington University.

Benoit, Jacques, Department of Histology, Medical School, Strasbourg, France.

Bissonnette, T. Hume, Professor of Biology, Trinity College.

Bogdanovitch, Sinisha B., Assistant in the Department of Pharmacology, School of Medicine, Belgrade, Yugoslavia.

Bozler, Emil, Assistant Professor of Physiology, Ohio State University.

Brachet, Jean, Rockefeller Fellow, Princeton University.

Bradley, H. C., Professor of Physiological Chemistry, University of Wisconsin.

Brisbin, G. W. F., University of Manitoba.

Bronfenbrenner, J., Professor of Bacteriology, Washington University School of Medicine.

Brown, Dugald E. S., Assistant Professor of Physiology, New York University Medical College.

Buck, John B., National Research Fellow, California Institute of Technology.

Cable, Raymond M., Assistant Professor of Parasitology, Purdue University.

Calkins, Gary N., Professor of Protozoölogy, Columbia University.

Cameron, John A., Instructor, University of Missouri.

Cannan, R. Keith, Professor of Chemistry, New York University Medical College.

Carothers, E. Eleanor, Research Associate, University of Iowa.

Catcheside, David E., Lecturer, Botany School, Cambridge University, London, England.

Chambers, Robert, Research Professor of Biology, Washington Square College, New York University.

Cheney, Ralph H., Chairman and Professor of Biology, Long Island University.

Chidester, Floyd E., Professor of Zoölogy, Extension Division, College of Forestry, Syracuse University.

Child, George P., Instructor in Biology, Amherst College.

Clark, Eleanor Linton, University of Pennsylvania, School of Medicine.

Clark, Eliot R., Professor of Anatomy and Director, Department of Anatomy, University of Pennsylvania, School of Medicine.

Clowes, George H. A., Director of Research, Lilly Research Laboratories.
Cohen, Arthur, Seessel Research Fellow, Yale University.
Coker, R. E., Professor of Zoölogy, University of North Carolina.
Cole, Kenneth S., Assistant Professor of Physiology, College of Physicians and Surgeons, Columbia University.
Conklin, Edwin G., Professor Emeritus of Biology, Princeton University.
Coonfield, Benjamin R., Assistant Professor, Brooklyn College.
Copeland, Manton, Professor of Biology, Bowdoin College.
Corey, H. Irene, Research Associate, University of Pennsylvania.
Co Tui, Frank W., Associate Professor of Experimental Surgery, New York University Medical College.
Cowdry, E. V., Professor of Cytology, Washington University.
Cox, Edward H., Professor of Chemistry, Swarthmore College.
Croasdale, Hannah T., Research Assistant in Physiology, Dartmouth College.
Crowell, Sears, Instructor in Zoölogy, Miami University.
Curwen, Alice O., Assistant Professor of Anatomy, Woman's Medical College of Pennsylvania.
Dahl, A. Orville, Teaching Assistant, University of Minnesota.
David, Paul R., Columbia University.
Davson, Hugh, Rockefeller Fellow, University of London, London, England.
Denny, Martha, Instructor in Zoölogy, Connecticut College.
Diller, William F., Assistant Professor, Dartmouth College.
Donaldson, Henry H., Member, Wistar Institute.
Dorris, Frances, Research Asssitant in Embryology, Yale University.
Dreyer, William A., Instructor in Zoölogy, University of Cincinnati.
Drouet, Francis, Research Fellow, Yale University.
DuBois, Eugene T., Professor of Medicine, Cornell University Medical College.
Duggar, B. M., Professor of Physiological and Economic Botany, University of Wisconsin.
Failla, G., Physicist, Memorial Hospital, New York City.
Fenn, W. O., Professor of Physiology, University of Rochester, School of Medicine and Dentistry.
Ferguson, J. K. W., Assistant Professor of Physiology, Ohio State University.
Fischer, Ernst, Associate Professor of Physiology and Pharmacology, Medical College of Virginia.
Fisher, Kenneth C., Instructor in Zoölogy, University of Maine.
Frisch, John A., Professor of Biology, Canisius College.
Fry, Henry J., Visiting Investigator, Cornell University Medical College.
Garrey, W. E., Professor of Physiology, Vanderbilt University School of Medicine.
Gaylor, John B., Rockefeller Travelling Fellow, Rockefeller Foundation.
Goodrich, H. B., Professor of Biology, Wesleyan University.
Gottschall, Gertrude, Assistant, Cornell University Medical College.
Grant, Ronald, Assistant Lecturer in Zoölogy, University of Leeds, England.
Grave, Caswell, Professor of Zoölogy, Washington University.
Gregersen, Magnus I., Professor of Physiology, University of Maryland, School of Medicine.
Hadley, Charles E., Associate Professor of Biology, Montclair State Teachers' College.
Hadorn, Ernst, Rockefeller Foundation Fellow, Zoölogical Institute, Berne, Switzerland.
Hamburger, Viktor, Assistant Professor, Washington University.
Harvey, E. Newton, Professor of Physiology, Princeton University.
Harvey, Ethel Browne, Princeton University.
Haywood, Charlotte, Associate Professor of Physiology, Mount Holyoke College.

Heilbrunn, L. V., Associate Professor of Zoölogy, University of Pennsylvania.
Hendee, Esther C., Instructor in Biology, Russell Sage College.
Henshaw, Paul S., Biophysicist, Memorial Hospital, New York City.
Hess, Walter N., Professor of Biology, Hamilton College.
Hibbard, Hope, Professor, Oberlin College.
Hiestand, William A., Assistant Professor of Physiology, Purdue University.
Hill, Samuel E., Assistant in General Physiology, Rockefeller Institute.
Höber, Rudolf, Visiting Professor of Physiology, University of Pennsylvania.
Hodge, Charles, 4th, Instructor, Temple University.
Hoffman, Olive, Instructor in Medicine, University of Pennsylvania.
Hollaender, Alexander, Special Investigator, University of Wisconsin.
Holmes, Joseph H., Department of Physiology, University of Maryland, School of Medicine.
Hopkins, Dwight L., Assistant Professor, Duke University.
Howe, H. E., Editor, Industrial and Engineering Chemistry.
Howell, Charles DeWitt, Graduate Student, Johns Hopkins University.
Hughes, Elizbeth Drumtra, Instructor in Biology, Wilson College.
Hughes, Roscoe D., Teaching Assistant, Columbia University.
Hunninen, Arne V., Head of Biology Department, Oklahoma City University.
Hunter, George W., III, Assistant Professor of Biology, Wesleyan University.
Hutchings, Lois M., Teacher of Biology, Weequahic High School, Newark, N. J.
Irving, Laurence, Professor of Biology, Swarthmore College.
Jacobs, Merkel H., Professor of General Physiology, University of Pennsylvania.
Jenkins, George B., Professor of Anatomy, George Washington University.
Johlin, J. M., Associate Professor of Biochemistry, Vanderbilt University School of Medicine.
Johnson, Frank H., Eli Lilly and Company Research Fellow, Princeton University.
Jones, E. Ruffin, Jr., Associate Professor of Biology, College of William and Mary.
Jones, Ruth McClung, Instructor in Botany and Zoölogy, Swarthmore College.
Keil, Elsa M., Assistant Professor of Zoölogy, New Jersey College for Women.
Keltch, Anna K., Research Chemist, Eli Lilly and Company.
Keyes, Donald B., Professor of Chemical Engineering, University of Illinois.
Kidder, George W., Instructor in Biology, College of the City of New York.
Kille, Frank R., Assistant Professor of Zoölogy, Swarthmore College.
Kindred, James E., Associate Professor of Histology and Embryology, University of Virginia.
Knower, Henry McE., Research Associate in Biology, Yale University.
Knowlton, Frank P., Professor of Physiology, Syracuse University, College of Medicine.
Kopac, M. J., Research Associate in Biology, Washington Square College, New York University.
Korr, Irvin M., Instructor, New York University Medical College.
Krahl, Maurice E., Research Chemist, Eli Lilly and Company.
Kreezer, George, Research Associate, The Training School, Vineland, New Jersey.
Krieg, Wendell J. S., Instructor in Anatomy, New York University, Medical College.
Lancefield, D. E., Associate Professor of Zoölogy, Columbia University.
Lancefield, Rebecca C., Associate, Rockefeller Institute.
Levine, Darwin S., Teacher, Theodore Roosevelt High School, New York.
Lillie, Frank R., Professor of Embryology, Emeritus, The University of Chicago.
Lillie, Ralph S., Professor of General Physiology, The University of Chicago.

Loeb, Leo, Professor Emeritus of Pathology, Washington University, School of Medicine.
Loeffler, Louis, Professor of Pathology, Middlesex College.
Lucas, Alfred M., Associate Professor, Iowa State College.
Lucas, Miriam Scott, Iowa State College.
Luyet, Basile J., Associate Professor of Biology, St. Louis University.
Lynn, W. Gardner, Instructor in Zoölogy, Johns Hopkins University.
McClung, Clarence E., Director, Zoölogical Laboratory, University of Pennsylvania.
Macdougald, Thomas J., Rockefeller Foundation Fellow in Experimental Cytology, Trinity College, Dublin, Ireland.
Maes, Julien, C. R. B., Fellow, Harvard University Medical School.
Magruder, S. R., Assistant in Anatomy, Cornell University Medical College.
Marsland, Douglas, Assistant Professor of Biology, Washington Square College, New York University.
Martin, W. E., Instructor in Biology, Purdue University.
Mathews, Albert P., Professor of Biochemistry, University of Cincinnati.
Matthews, Samuel A., Associate in Anatomy, University of Pennsylvania.
Mazia, Daniel, National Research Fellow, Princeton University.
Menkin, Valy, Instructor in Pathology, Harvard University Medical School.
Michaelis, Leonor, Member, Rockefeller Institute.
Miller, Faith Stone, Ohio University.
Miller, James A., Instructor in Zoölogy, Ohio University.
Mitchell, Philip H., Professor of Physiology, Brown University.
Morgan, Lilian V., Pasadena, California.
Morgan, T. H., Professor of Experimental Zoölogy, California Institute of Technology.
Morrill, Charles V., Associate Professor of Anatomy, Cornell University Medical College.
Morris, Samuel, Research Zoölogist, Temple University.
Murlin, John R., Professor of Physiology, University of Rochester.
Nebel, Bernard R., Associate in Research, Agricultural Experiment Station, Geneva, New York.
Netting, M. Graham, Curator of Herpetology and Lecturer in Biology, Carnegie Museum and University of Pittsburgh.
Newton, W. H., Reader in Physiology, University of London, England.
Nonidez, José F., Assistant Professor of Anatomy, Cornell University Medical College.
Northrop, John H., Rockefeller Institute for Medical Research.
Olson, Magnus, Research Assistant, University of Minnesota.
Orr, Paul R., Instructor, Brooklyn College.
Osterhout, W. J. V., Member, Rockefeller Institute for Medical Research, New York City.
Packard, Charles, Assistant Professor of Zoölogy, Columbia University.
Painter, Elizabeth S., Instructor in Physiology, University of Maryland, School of Medicine.
Parker, G. H., Professor of Zoölogy Emeritus, Harvard University.
Parmenter, Charles L., Associate Professor of Zoölogy, University of Pennsylvania.
Parpart, Arthur K., Assistant Professor of Physiology, Princeton University.
Patrick, Ruth, Professor of Botany, Pennsylvania School of Horticulture.
Pierce, Madelene E., Instructor, Vassar College.
Plough, Harold H., Professor of Biology, Amherst College.
Pollister, Arthur W., Assistant Professor of Zoölogy, Columbia University.
Potter, Truman S., Research Associate, The University of Chicago.
Poulson, Donald F., Instructor in Zoölogy, Yale University.
Prescott, G. W., Assistant Professor of Biology, Albion College.

Prosser, C. Ladd, Assistant Professor of Physiology, Clark University.
Rankin, John S., Jr., Teaching Fellow, Amherst College.
de Renyi, George S., Associate Professor of Anatomy, University of Pennsylvania, School of Medicine.
Richards, Oscar W., Instructor in Biology, Osborn Zoölogical Laboratory, Yale University.
Ritchie, Lawrence S., Instructor in Biology, University of North Carolina Women's College.
Robeson, John M., Instructor in Zoölogy, Syracuse University.
Root, Raymond W., Assistant Professor of Biology, College of the City of New York.
Root, Walter S., Associate Professor of Physiology, University of Maryland School of Medicine.
Ruebush, Trenton K., Instructor, Yale University.
Rugh, Roberts, Instructor in Zoölogy, Hunter College.
Russell, Alice M., Instructor, University of Pennsylvania.
Salomon, Kurt, Honorary Research Fellow, Yale University.
Sauer, Mary E., Instructor in Anatomy, University of Alabama.
Sayles, Leonard P., Assistant Professor of Biology, College of the City of New York.
Schaeffer, A. A., Chairman, Department of Biology, Temple University.
Scharrer, Berta, Research Assistant, Neurologisches Institute, Frankfurt, Germany.
Scharrer, Ernst, Research Fellow, Neurologisches Institute, Frankfurt, Germany.
Schechter, Victor, Instructor, College of the City of New York.
Schmidt, Ida Genther, Assistant Professor of Anatomy, University of Cincinnati, College of Medicine.
Schmidt, L. H., Research Fellow in Biochemistry, Christ Hospital, and University of Cincinnati College of Medicine.
Schmitt, Francis O., Associate Professor of Zoölogy, Washington University.
Schmitt, Otto H., Washington University.
Schotté, Oscar E., Assistant Professor of Biology, Amherst College.
Schram, Mildred W. S., International Cancer Research Foundation.
Scott, Allan C., Instructor in Biology, Union College.
Scott, Sister Florence Marie, Professor of Zöology, Seton Hill College.
Sembrat, Kazimierz, Research Fellow in the Natural Sciences, University of Lwow, Poland.
Sembrat, Zofja, Adjunct at the Laboratory of Histology and Embryology, Academy of the Veterinary Medicine, Lwow, Poland.
Shannon, James A., Assistant Professor of Physiology, New York University Medical College.
Shapiro, Herbert, Research Assistant in Physiology, Princeton University.
Shaw, Myrtle, Senior Bacteriologist, New York State Department of Health.
Shedlovsky, Theodore, Associate, Rockefeller Institute.
Shumway, Waldo, Professor of Zoölogy, University of Illinois.
Sichel, Ferdinand J. M., Instructor in Physiology, Howard University School of Medicine.
Slifer, Eleanor H., Associate in Zoölogy, State University of Iowa.
Smith, Dietrich C., Instructor in Physiology, University of Tennessee, College of Medicine.
Solandt, Donald Y., Assistant Professor of Physiology, University of Toronto.
Sonneborn, Tracy M., Associate in Zoölogy, Johns Hopkins University.
Spencer, Warren P., Professor of Biology, College of Wooster.
Steinbach, Henry B., Instructor in Zoölogy, University of Minnesota.
Stern, Kurt G., Visiting Lecturer of Physiological Chemistry, Yale University.
Stewart, Dorthy R., Associate Professor of Biology, Skidmore College.

Stockard, Charles R., Professor of Anatomy, Cornell University Medical College.
Stunkard, H. W., Professor of Biology, New York University.
Sturtevant, Alfred H., Professor of Genetics, California Institute of Technology.
Summers, Francis M., Instructor in Biology, Bard College, Columbia University.
Taft, Charles H., Jr., Associate Professor of Pharmacology, University of Texas, Medical Branch.
Tartar, Vance, Assistant in Biology, Yale University.
Tashiro, Shiro, Professor of Biochemistry, University of Cincinnati, College of Medicine.
Taylor, William Randolph, Professor of Botany, University of Michigan.
Terni, Tullio, Professor of Histology-Embryology and Anatomy and Director of the Institute of Anatomy, University of Padua, Italy.
Thornton, C. S., Assistant Professor of Biology, Kenyon College.
Tittler, Irving A., Instructor in Biology, Brooklyn College.
Törö, Emeric, Assistant Professor, Anatomical Institution of University of Debreceu.
Trager, William, Assistant, Rockefeller Institute.
Turner, Abby H., Professor of Physiology, Mount Holyoke College.
Tyler, Albert, Instructor in Embryology, California Institute of Technology.
Uhlenhuth, Eduard, Professor of Anatomy, University of Maryland, School of Medicine.
Vicari, Emilia M., Associate in Anatomy, Cornell University Medical College.
Walker, Roland, Instructor in Biology, Rensselaer Polytechnic Institute.
Warren, Marshall R., Graduate Assistant in Zoölogy, University of Cincinnati.
Waterman, A. J., Assistant Professor of Biology, Williams College.
Weissenberg, Richard, Visiting Professor of Cytology, Washington University.
Wenrich, D. H., Professor of Zoölogy, University of Pennsylvania.
Whiting, Anna R., Guest Investigator, University of Pennsylvania.
Whiting, P. W., Associate Professor of Zoölogy, University of Pennsylvania.
Wichterman, Ralph, Instructor, Temple University.
Willier, B. H., Head, Division of Biological Sciences, University of Rochester.
Wilson, Edmund B., Professor Emeritus in Residence, Columbia University.
Wilson, May G., Assistant Professor of Pediatrics, Cornell University Medical College.
Wolf, E. Alfred, Associate Professor of Biology, University of Pittsburgh.
Wolf, Ernst, Harvard University.
Wolf, Opal M., Assistant Professor, Goucher College.
Woodruff, Lorande L., Professor of Protozoölogy, Yale University.
Woodward, Alvalyn E., Assistant Professor of Zoölogy, University of Michigan.
Worley, Leonard G., Instructor, Brooklyn College.
Yntema, C. L., Assistant Professor, Cornell University Medical College.
Young, Allan C., University of Rochester, Medical School.
Young, Dixie, Yale University.
Zweifach, Benjamin W., Research Assistant, New York University.

Beginning Investigators

Alley, Armine, Research Assistant, McGill University.
Andersch, Marie, Assistant Professor, Woman's Medical College, Philadelphia.
Ballentine, Robert, Junior Fellow in Biology, Princeton University.
Barry, Alexander, Harvard University.
Bauer, Karl, University of Berlin.
Black, Edgar C., Demonstrator in Experimental Biology, University of Toronto.
Bradley, E. Morton, Assistant, University of Rochester.

Brehme, Katherine S., Editorial Assistant, "Genetics," Columbia University.
Briggs, R. W., Assistant in Biology, Harvard University.
Brill, Edmund R., Graduate work in Biology, Harvard University.
Cable, Mary Tupper, Graduate Student, Purdue University.
Churney, Leon, Instructor in Zoölogy, University of Pennsylvania.
Claff, C. Lloyd, 5 Van Beal Road, Randolph, Massachusetts.
Cleverdon, Maude A., Student, Oberlin College.
Commoner, Barry, Graduate Student, Columbia University.
Conley, Carroll L., Teaching Fellow in Physiology, University of Maryland, School of Medicine.
Cornman, Ivor, Teaching Fellow, Washington Square College, New York University.
Corson, Samuel, Research Assistant in Cell Physiology, Washington Square College, New York University.
Culbreth, Sarah E., Graduate Fellow, Duke University.
Derrickson, Mary B., Graduate Assistant, Duke University.
Dethier, Vincent G., Graduate Student, Harvard University.
Dienes, Priscilla A., Student, Radcliffe College.
Donnellon, James A., Student, University of Pennsylvania.
Frank, John A., Yale University.
Guttman, Rita, Graduate Student in Physiology, College of Physicians and Surgeons, Columbia University.
Harman, Doris Spier, Assistant Teacher of Biology, Straubenmüller Textile High School.
Harris, Daniel, Instructor of Zoölogy, University of Pennsylvania.
Hawley, Katharine J., Student, Public Health Department, Yale University.
Hiatt, Edwin P., Graduate Assistant, Duke University.
Hinchey, M. Catherine, Graduate Student, University of Pennsylvania.
Hobson, Lawrence B., Graduate Assistant in Zoölogy, University of Cincinnati.
Hollingsworth, Josephine, Graduate Student, University of Pennsylvania.
Hornor, Helen Betty, Assistant Teacher, Barnard College, Columbia University.
Hunter, Laura N., Instructor, Pennsylvania College for Women.
Ives, Philip T., Research Fellow in Biology, Amherst College.
Kaliss, Nathan, Assistant in Zoölogy, Columbia University.
Levenson, Alfred S., Graduate Student, University of Pittsburgh.
Lichtman, Frieda, New York University.
Lipman, Harry J., Graduate Assistant, University of Pittsburgh.
McAllister, Ferdinand F., Research Fellow, University of Maryland, School of Medicine.
Malick, Leona F., Graduate Student, Brown University.
Malone, Edna J., Teacher of Biology, Walton High School.
Maxwell, Jane, Instructor, Carnegie Institute of Technology.
Mayo, Virginia, Graduate Student, Radcliffe College.
Moore, Caroline, Graduate Student, University of Pennsylvania.
Moore, John A., Assistant in Zoölogy, Columbia University.
Moore, Walter G., Graduate Assistant, University of Minnesota.
Morgan, Isabel M., Graduate Student, University of Pennsylvania.
Moser, Floyd, Graduate Student, University of Pennsylvania.
Neel, James, Graduate Teaching Assistant, University of Rochester.
Novikoff, Alex B., Tutor, Brooklyn College.
Patterson, John L., Jr., Student of Medicine, Medical College of Virginia.
Peabody, Elizabeth B., Graduate Student, Radcliffe College.
Pierson, Bernice F., Graduate Student, Johns Hopkins University.
Porter, Keith R., Assistant, Harvard University.
Ricci, Nelda I., Laboratory Assistant, Rockefeller Foundation.
Rose, S. Meryl, Teaching Assistant, Columbia University.

Rosenbusch, Carlos, Graduate Student, Iowa State College.
Rosenfield, Richard, Medical Student, University of Pittsburgh.
Safford, Virginia, Laboratory Technician, Wellesley College.
Sawin, Horace J., Assistant, Harvard University.
Schenthal, Joseph E., Weaver Research Fellow in Anatomy, University of Maryland, School of Medicine.
Schoenborn, Henry W., Graduate Assistant, New York University.
Smith, Jay Alfred, Assistant in the Department of Zoölogy, Johns Hopkins University.
Smith, R. Dale, Graduate Assistant, University of Pittsburgh.
Snider, Ray S., Research Assistant, Washington University.
Solberg, Archie N., University Fellow, Columbia University.
Stoudt, Harry N., Graduate Student, Johns Hopkins University.
Ting, T. P., Graduate Student, University of Pennsylvania.
Trombetta, Vivian, Assistant in Botany, Barnard College, Columbia University.
Uhrich, Jacob, Fellow, The University of Chicago.
Voter, Muriel A., Wheaton College.
Wilbur, Karl M., Fellow, University of Pennsylvania.
Wilhelmi, Raymond W., Graduate Assistant, New York University.
Worley, Elizabeth K., Lecturer, Barnard College, Columbia University.
Young, Roger A., Assistant Professor in Zoölogy, Howard University.
Zwilling, Edgar, Assistant in Zoölogy, Columbia University.

Research Assistants

Algire, Glenn H., Weaver Fellow in Anatomy, University of Maryland, School of Medicine.
Armstrong, Louise Schmuck, Cornell University Medical College.
Beck, Lyle V., Research Fellow, University of Pennsylvania.
Buchheit, J. Robert, Research Assistant, Eli Lilly and Co.
Castle, Ruth M., Student, Montclair State Teachers College.
Chambers, Alfred H., Jr., Graduate Student, University of Pennsylvania.
Chambers, Edward L., Student, Princeton University.
Copeland, D. Eugene, Assistant in Biology, Amherst College.
Crystal, Sidney, Research Assistant, Rockefeller Institute.
Curtis, Howard J., Associate in Physiology, College of Physicians and Surgeons, Columbia University.
Dugal, Louis Paul, Instructor, University of Montreal.
Dziemian, Arthur J., Fellow, Princeton University.
Eggleston, Nancy M., Student, Mount Holyoke College.
Etkin, William, Instructor, College of the City of New York.
Fien, Irving, Trinity College.
Foerster, James, Washington University.
Fraser, Doris A., Fellow, University of Pennsylvania.
Freeman, A. M., Jr., Student, Vanderbilt University, School of Medicine.
Gehenio, Sister Pierre M., Graduate Assistant in Biology, St. Louis University.
Glassman, Harold N., Graduate Student, University of Pennsylvania.
Goldin, Abe, Assistant, Brooklyn College.
Grand, C. G., Research Associate, Washington Square College, New York University.
Hamdi, Turgut N., Student, University of Pennsylvania.
Hershkowitz, S. G., Student, New York University Medical College.
Hill, Edgar S., Instructor in Biochemistry, Washington University, School of Medicine.
Höber, Josephine, Philadelphia, Pennsylvania.
Hodapp, Eugene L., Graduate Fellow in Biology, St. Louis University.
Horowitz, Norman, Teaching Fellow, California Institute of Technology.

Horvath, Steven M., Ohio State University.
Hummel, Katharine P.
Hutchens, John, Graduate Assistant, Johns Hopkins University.
Jailer, Joseph W., Assistant, College of Physicians and Surgeons, Columbia University.
Kalmanson, George M., Research Assistant, Washington University.
Keefe, Eugene L., Research Assistant, Washington University.
Korr, Renée B., New York University Medical College.
Kritzler, Henry, Student, Bard College, Columbia University.
Law, Lloyd W., Assistant, Harvard University.
Lewis, Lena A., Research Assistant, Ohio State University.
Lyon, Rhea C., Research Assistant, University of Maryland, School of Medicine.
Moore, Gordon F., Student, Washington University, School of Medicine.
Morris, Marion C., Instructor, Washington University, School of Medicine.
Mundt, Henry, Jr., Student, Amherst College.
Nicoll, Paul A., Fellow Biological Sciences, National Research Council.
O'Brien, John P., Johns Hopkins University.
Öhnell, Richard F., Kar Institute, Stockholm, Sweden.
Rawles, Mary E., Research Assistant, University of Rochester.
Ricca, Renato A., Student, University of Pennsylvania, School of Medicine.
Riehl, Doris, Graduate Student, Yale University.
Robertson, Mrs. C. W., Research Assistant, New York University.
Rubin, Saul H., Research Assistant, New York University.
Salk, Jonas, Christian A. Herter Fellow, New York University Medical College.
Shaw, Isidor, Technician in Biology, Long Island University.
Simmons, Eric L., Student, Swarthmore College.
Smith, Carl C., Research Assistant, University of Cincinnati.
Spencer, Joseph M., Research Assistant, College of Physicians and Surgeons, Columbia University.
Trost, Mrs. Thelma, Assistant, University of Pennsylvania.
Wagner, Carroll E., Research Assistant, University of Maryland, School of Medicine.
Warner, Robert C., Teaching Fellow, New York University Medical College.
Weisberg, Harry F., Fellow in Biology, College of the City of New York.
Wightman, John C., Assistant, Brown University.
Wolf, Fred T., Research Assistant, University of Wisconsin.
Young, Saul B., Rockefeller Institute.

Students

BOTANY

Donovan, Mary K., Teacher of Biology, Boston Teachers College.
Hicks, Jaqueline P., Barnard College.
Hilgeman, Dorothea E., Goucher College.
Kalter, Louis B., Student, Antioch College.
Lloyd, Frances V., University of Alabama.
Moseley, Charles B., Dartmouth College.
Runk, Benjamin F. D., Service Fellow, University of Virginia.
Stevenson, Barbara, Student, Wellesley College.
Travis, Mildred T., University of Pennsylvania.

EMBRYOLOGY

Alsup, Fred W., Professor of Zoölogy, Morristown College.
Angell, Nancy, Bryn Mawr College.
Blanc, Richard, Graduate Assistant in Biology, Amherst College.

Bozeman, Martha L., Graduate Student, University of Illinois.
Christhilf, Stuart M., Jr., Student, St. John's College.
Cleverdon, Maude A., Student, Oberlin College.
Cornman, Ivor, Teaching Fellow, Washington Square College, New York University.
Dienes, Priscilla A., Radcliffe College.
Fogel, Paul J., 2 MacKenzie Road, Morristown, New Jersey.
Friedman, Sam, Student, Washington Square College, New York University.
Griffiths, Raymond B., Student, University of Rochester.
Gross, Charlotte C., Montclair State Teachers' College.
Hatch, Cleora, Radcliffe College.
Kellogg, Margaret P., Student, Smith College.
Kotcher, Emil, Graduate Assistant, Wesleyan University.
Kraft, Jean C., Northwestern University.
Leonard, Barbara H., Undergraduate Assistant, Oberlin College.
Logan, Arch H., Jr., Student, Washington and Jefferson College.
Mackenzie, Malcolm B., Student, McGill University.
Milford, John J., Graduate Assistant, New York University.
Molter, John A., Instructor, University of Notre Dame.
Moore, Walter G., Graduate Assistant, University of Minnesota.
Moorman, Albert E., Zoölogy Assistant, Northwestern University.
Morgan, Gwendolynn W., Science Teacher, Rosemarie Junior School.
Neel, James V., Teaching Assistant, University of Rochester.
Nichols, Florence E., DePauw University.
Smith, Elizabeth T., Assistant in Zoölogy, Northwestern University.
Smith, Marian, Student, Washington University.
Streett, J. Clark, Jr., Princeton University.
Uhrich, Jacob, Fellow, The University of Chicago.
White, Elizabeth L., Goucher College.
Williams, Charles F., Laboratory Assistant, DePauw University.
Williams, Charles R., Graduate Student, University of Illinois.
Wright, Elsie Star, Graduate Student, Johns Hopkins University.
Wrightington, Margaret, Student, Vassar College.

PHYSIOLOGY

Black, Edgar C., Demonstrator in Experimental Biology, Toronto University.
Bridgman, Jane, Graduate Student, Yale University.
Culbreth, Sarah E., Graduate Fellow, Duke University.
Evans, John David, Graduate Assistant, Northwestern University.
Farney, Esther, Student, University of Kansas.
Fetcher, Edwin S., Jr., Rockefeller Institute.
Grant, Wilson C., Wesleyan University.
Hiatt, Edwin P., Graduate Assistant, Duke University.
Hock, Charles W., Harrison Fellow, University of Pennsylvania.
Huntington, Margaret O., Washington University, School of Medicine.
Lambert, Barbara Chase, Graduate Assistant in Physiology, Mount Holyoke College.
Nebel, Bernard R., Research Assistant, New York State Agricultural Experimental Station.
Patterson, John L., Jr., Student, Medical College of Virginia.
Ravitz, Louis A., University of Wisconsin.
Safford, Virginia, Laboratory Technician, Wellesley College.
Smith, Elizabeth W., Oberlin College.

PROTOZOÖLOGY

Belcher, Jane C., Columbia University.
Brooke, Marion M., Teacher, Emory Junior College.
Caldwell, John J., Teacher, Grand Falls High School.
Chasanoff, Ida, Student, Columbia University.
Dropkin, Victor H., Graduate Student, The University of Chicago.
Fippinger, Esther, Hunter College.
Green, Susan A., Professor of Biology, Maryville College.
Gurley, Mary K., Laboratory Assistant in Bacteriology, College of Physicians and Surgeons.
Hartwell, Mary A., 75 College Street, Brockport, New York.
Loukides, James, Student, Rutgers University.
Meigs, John F., Jr., Harvard University.
Noyes, Barbara C., Student, Columbia University.
Schlossman, Abraham, 295 Pennsylvania Avenue, Brooklyn, New York.
Snider, Hester B., Columbia University.
Voter, Muriel A., Wheaton College.
White, Elizabeth C., Columbia University.

INVERTEBRATE ZOÖLOGY

Albrink, Wilhelm S., Student, Oberlin College.
Bailey, Villa E., Oberlin College.
Baird, Isabelle, Graduate Assistant, Mount Holyoke College.
Barden, Albert A., Jr., Northwestern University.
Barkley, Mabel A., Teacher, Central High School, Washington, D. C.
Berry, Charles M., Laboratory Instructor, DePauw University.
Bookhout, Cazlyn G., Instructor in Zoölogy, Duke University.
Bottom, Curtis H., Assistant Professor, Centre College.
Bridgman, Josephine, Teacher of Zoölogy, Flora Macdonald College.
Burbanch, William D., Instructor in Biology, Earlham College.
Carroll, William R., Student, Swarthmore College.
Culberson, Mabel H., Simmons College.
Dana, Harriet M., Student, Swarthmore College.
Dvoskin, Samuel, College of the City of New York.
Eisenberg, Esther, Laboratory Assistant, Eastern District High School.
Evans, Hiram J., Hamilton College.
Fink, Harold K., Student, Princeton University.
Gilman, Lauren C., Graduate Student, Johns Hopkins University.
Goodchild, Chaundey G., Graduate Assistant, New York University.
Goodline, Mary A., Student, Montclair State Teachers' College.
Gortner, Ross A., Jr., Instructor in Biology, Wesleyan University.
Griffin, Donald R., Harvard University.
Hamilton, Howard L., State University of Iowa.
Harrold, Charles M., Wabash College.
Haskin, Harold H., Assistant, Harvard University.
Holthausen, Carl F., Jr., Assistant in Biology, Amherst College.
Johnson, Marian, Student, American University.
Kuhn, Evelyn M., Research Assistant, Rice Institute.
Kurtz, Elizabeth L., Wilson College.
McBride, Margaret P., Student, Pennsylvania College for Women.
Mapp, Frederick E., Teacher, Atlanta University.
Marrow, Lucille, High School Biology Teacher.
Martindale, Florence M., Instructor in Zoölogy, Mount Holyoke College.
Merwin, Ruth M., Mount Holyoke College.

LIBRARY

Miller, Albert, Instructor in Entomology, Cornell University.
Müller, Mrs. William A., Graduate Student, University of Minnesota.
Müller, William A., Teaching Assistant, University of Minnesota.
O'Brien, John P., Graduate Student, Johns Hopkins University.
Orange, Jeannette, Graduate Student, Columbia University.
Osterud, Kenneth L., Graduate Assistant in Biology, New York University.
Perry, Dorothy F., Smith College.
Ramsey, Helen J., 242 Connolly Street, West Lafayette, Indiana.
Reed, Eleanor, Technician, University of Pittsburgh.
Rockstein, Morris, Student, Brooklyn College.
Ryan, Francis J., Assistant in Zoölogy, Columbia University.
Sawin, Horace J., Assistant, Harvard University.
Schneider, Ruth M., Skidmore College.
Scrimshaw, Neven S., Student, Assistant in Zoölogy, Ohio Wesleyan University.
Stearns, John B., Assistant, Amherst College.
Steele, Dewey G., Assistant Professor of Genitics, Connecticut State College.
Strandine, Eldon J., Northwestern University.
Sugarbaker, John D., Student, New Jersey State Teachers College.
Swanson, Carl P., Student, Massachusetts State College.
Walker, Sally, Radcliffe College.
Ward, Elizabeth B., Graduate Assistant in Zoölogy, University of Cincinnati.
Williams, Myra A., Graduate Assistant in Zoölogy, Duke University.
Wilson, James G., Graduate Assistant, University of Richmond.

3. TABULAR VIEW OF ATTENDANCE

	1933	1934	1935	1936	1937
Investigators—Total	319	323	315	359	391
Independent	210	222	208	226	256
Under Instruction	66	49	56	76	74
Research Assistants	43	52	51	57	61
Students—Total	118	131	130	138	133
Zoölogy	54	54	55	55	57
Protozoölogy	11	11	16	17	16
Embryology	28	30	33	34	35
Physiology	19	23	20	22	16
Botany	6	13	6	10	9
Total Attendance	437	454	445	497	524
Less Persons registered as both students and investigators	12	15	16	24	13
	425	439	429	473	511
Institutions Represented—Total	120	131	143	158	165
By Investigators	92	98	111	120	134
By Students	58	75	70	77	79
Schools and Academies Represented					
By Investigators	1	1	–	2	3
By Students	2	5	3	3	2
Foreign Institutions Represented					
By Investigators	5	4	7	9	16
By Students	–	1	1	5	–

4. SUBSCRIBING AND COÖPERATING INSTITUTIONS IN 1937

American University
Amherst College
Atlanta University
Bowdoin College
Brown University
Bryn Mawr College
California Institute of Technology
College of Charleston
College of Physicians and Surgeons
Columbia University
Cornell University
Cornell University Medical College
C. R. B. Educational Foundation, Inc.
Dartmouth College
DePauw University
Duke University
General Education Board
Goucher College
Hamilton College
Harvard University
Hunter College
Industrial & Engineering Chemistry, of the American Chemical Society
Iowa State College
Johns Hopkins University
Kenyon College
Eli Lilly & Co.
Long Island University
Massachusetts State College
Medical College of Virginia
Memorial Hospital, New York City
Morehouse College
Mount Holyoke College
New York State Department of Health
New York University
New York University Medical School
Northwestern University
Oberlin College
Pennsylvania College for Women
Princeton University
Purdue University
Radcliffe College
Rice Institute
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
St. John's College
Seton Hill College
Smith College
State University of Iowa
Swarthmore College
Syracuse University
Temple University
Union College
University of Chicago
University of Cincinnati
University of Georgia, School of Medicine
University of Illinois
University of Maryland Medical School
University of Minnesota
University of Pennsylvania
University of Pennsylvania Medical School
University of Pittsburgh
University of Rochester
University of Rochester Medical School
University of Virginia
University of Wisconsin
Vanderbilt University Medical School
Vassar College
Wabash College
Washington University
Washington University Medical School
Wellesley College
Wesleyan University
Wheaton College
Wilson College
Wistar Institute of Anatomy and Biology
Yale University

5. EVENING LECTURES, 1937

Tuesday, June 29	Dr. W. T. Astbury	"The X-ray Interpretation of Protein Structure."
Friday, July 2	Dr. H. B. Goodrich	"The Development of Inherited Color Patterns in Fish."
Friday, July 9	Dr. E. Newton Harvey	"Electrical Potentials from the Human Brain."
Friday, July 16	Dr. Alexander Hollaender	"Studies in the Problem of Mitogenetic Radiation."
Wednesday, July 21	Dr. Edgar Allen	"Ovulation in Mammals and Internal Secretions Involved."
Friday, July 23	Dr. Leonor Michaelis	"The Problem of the Oxidation of Organic Compounds and Its Catalysis under Biological Conditions."
Friday, July 30	Dr. Caswell Grave	"Metamorphosis in Ascidians."
Thursday, August 5	Dr. F. Gudernatsch	"The Thymus Problem: Its Present Status after 25 Years of Experimentation."
Friday, August 6	Dr. Ernst Fischer	"Some Physical Aspects of Muscular Contraction."
Friday, August 13	Dr. John H. Northrop	"Concentration and Purification of Bacteriophage."
Wednesday, August 18	Dr. Robert Cushman Murphy	"The Gates of the Antarctic."
Thursday, August 19	Mr. John S. Garth	"To Galapagos aboard Velero III"
Friday, August 20	Dr. H. C. Stetson	"The Submarine Canyons of the Continental Shelf."
Monday, August 30 (Under the joint auspices of the Genetics Society of America and the Marine Biological Laboratory)	Dr. Boris Ephrussi	"Aspects of the Physiology of Gene Action."

6. SHORTER SCIENTIFIC PAPERS, 1937

Tuesday, July 6	
Dr. Ethel Browne Harvey	"Parthenogenetic Merogony in the Naples Sea-urchins."
Dr. Jean Brachet	"Some Oxidative Properties of Isolated Amphibian Germinal Vesicles."
Dr. Albert Tyler	"Influence of Temperature and Other Agents on the Respiration and Development of Marine Eggs."
Tuesday, July 13	
Dr. Warren Andrew	"Effects of Fatigue Due to Muscular Exercise on the Purkinje Cells of the Cerebellum of Mice at Various Ages."
Mr. Zareh Hadidian, Mr. Milton S. Dunn and Dr. Roland Walker	"Localization in the Oculomotor Nuclei of the Goldfish."
Dr. Ernst Scharrer	"Some New Observations on the Secretory Activity of Neurons."
Dr. C. Ladd Prosser	"Synaptic Transmission in the Sixth Abdominal Ganglion of the Crayfish."
Tuesday, July 20	
Dr. L. G. Barth	"Chemical Stimulation of the Amphibian Ectoderm."
Dr. Viktor Hamburger	"Limb Bud Transplantation in Chick Embryos."
Dr. Oscar E. Schotté	"Adult Organizers and Their Action in Adult Tissues."
Dr. L. S. Stone	"The Development of a Salamander, Amblystoma punctatum," shown by motion pictures.
Tuesday, July 27	
Dr. J. K. W. Ferguson and Dr. F. A. Hitchcock	"The Effect of Standing on the Carbon Dioxide Content of Alveolar Air and Total Ventilation Volume."
Dr. Eugene F. DuBois and Dr. James D. Hardy	"The Mechanism of the Loss of Heat from the Human Body."
Dr. Emil Bozler	"Peripheral Inhibition of Smooth Muscle."
Dr. William R. Amberson, Dr. Thomas P. Nash, Dr. Arthur G. Mulder and Miss Dorothy Binns	"The Relationship of Tissue Chloride to Blood Chloride."

Monday, August 2

Dr. Ruth Patrick	"The Use of Diatoms from Geological Excavations at Clovis, New Mexico, as Indicators of Water Conditions."
Mr. H. N. Stoudt	"Gemmipary in Kalanchoe rotundifolia and Other Crassulaceae."
Mr. A. O. Dahl	"Pollen Analysis of the Air in Relation to Hay-fever."

Tuesday, August 3

Dr. P. W. Whiting	"A Convenient Test of Physical Agents as Producers of Dominant Lethals."
Dr. Bernard R. Nebel	"Cytological Observations on Colchicine."
Dr. R. Weissenberg	"Demonstration of Vital Staining Preserved in Paraffin Sections of Lamprey Embryos." (Bismark Brown Method.)
Dr. William R. Duryee	"Microfilm on Some Experiments on Isolated Amphibian Germinal Vesicles."
Dr. Robert Chambers	"Cortical Cytolysis of the Echinoderm Egg."

Tuesday, August 10

Dr. Francis M. Summers	"Some Aspects of Normal and Regulative Development in the Colonial Ciliate, Zoöthamnium alternans."
Dr. A. A. Schaeffer	"Morphology, Behavior and Reproduction in Type A and Type B of Chaos chaos Linnaeus, the Giant Multi-nucleate Ameoba of Roesel."
Dr. Theodor von Brand	"Observations upon the Chemical Composition and the Metabolism of a Larval Parasitic Nematode."
Dr. Paul S. Galtsoff	"Observations and Experiments on Sex Change in the Adult American Oyster, Ostrea virginica."

Tuesday, August 17

Dr. Albert Tyler and Mr. Norman H. Horowitz	"A Sea Water Buffer for Marine Eggs."
Mr. Edgar C. Black and Dr. Laurence Irving	"The Effect of CO_2 upon the Oxygen Capacity of the Blood of Some Fresh-Water Fish."
Dr. Irvin M. Korr	"Oxidative Mechanisms in the Resting and Fertilized Sea-urchin Egg."

Dr. Kurt G. Stern and
Mr. Delafield Dubois .... "Methods for the Study of Rapid Chemical Reactions and Their Application to the Kinetics of Enzyme-Substrate and Enzyme-Inhibitor Compound Formation."

Tuesday, August 24
Dr. B. Luyet .... "Mechanism of Cellular Death by Freezing."

Dr. D. Mazia .... "Binding and Penetration of Bivalent Cations in Elodea Cells."

Dr. G. H. A. Clowes,
Miss A. K. Keltch and
Dr. M. E. Krahl .... "Factors Governing Cellular Responses to Nitro and Halo Phenols."

Dr. M. E. Krahl,
Dr. G. H. A. Clowes and
Miss A. K. Keltch .... "The Possible Rôle of Acidic Dissociation in the Physiological Effects Produced by Nitro and Halo Phenols."

Dr. Rudolf Höber and
Dr. Bernard R. Nebel .... "Depolarization of Muscle and Nerve Membranes by Organic Substances."

Dr. A. E. Navez and
Dr. Ethel Browne Harvey ..... "Indophenol Oxidase in the Arbacia Egg and the Nadi Reagent."

Thursday, September 2
Dr. Theodore Shedlovsky ...... "The Ionization of Carbonic Acid."

7. GENERAL SCIENTIFIC MEETING, 1937

Thursday, August 26
Dr. B. J. Luyet and
Mr. E. L. Hodapp .... "On Some Conditions Determining Sub-cooling in Plant Tissues."

Dr. B. J. Luyet and
Sister P. M. Gehenio .... "On the Double Freezing Point of Some Living Tissues."

Dr. H. J. Curtis and
Dr. K. S. Cole .... "Transverse Electric Impedance of the Squid Giant Axon."

Dr. K. S. Cole and
Mr. J. M. Spencer .... "Electric Impedance of Suspensions of Unfertilized and Fertilized Arbacia Eggs."

Dr. K. S. Cole and Dr. H. J. Curtis	"Electric Impedance of Single Arbacia Eggs."
Dr. Samuel E. Hill	"The Effect of NaCl on Potentials in Nitella."
Dr. M. J. Kopac	"The Coalescence of a Plant Cell with Oil Drops."
Dr. Ernst Fischer	"The Influence of Length, Tension, and Tone upon the Birefringence of Smooth Muscles (Phascolosoma and Thyone)."
Mr. Samuel A. Corson	"The Mechanism of Salt Penetration in Amoeba—Some Micromanipulative Data."
Dr. Alexander Hollaender	"The Efficiency of Monochromatic Ultraviolet Radiation in the Activation of Arbacia Eggs."
Dr. Ethel Browne Harvey and Dr. Alexander Hollaender	"Activation of Centrifuged Whole Eggs of Arbacia and Their Fractions by Monochromatic Ultraviolet Radiation."
Dr. B. R. Nebel, Dr. E. B. Harvey and Dr. Alexander Hollaender	"The Cytology of Arbacia punctulata Activated by Monochromatic Ultraviolet Radiation."
Mr. John A. Frank	"The Relationship of Sperm Extracts to the Fertilization Reaction in Arbacia."
Dr. L. V. Heilbrunn and Mr. Karl M. Wilbur	"Stimulation and Nuclear Breakdown in the Nereis Egg."
Mr. Edward L. Chambers	"The Movement of the Egg Nucleus in Relation to the Sperm Aster in Echinarachnius."
Dr. Robert Chambers	"The Physical State of the Wall of the Furrow in a Dividing Cell."

Friday, August 27

Dr. A. Orville Dahl	"Chromosome Studies in Sundew (Drosera)."
Miss M. Catherine Hinchey	"Mitosis in the Giant Amoeba, Chaos chaos Linnaeus."
Dr. James A. Miller	"Some Effects of Oxygen on Polarity in Tubularia crocea."
Dr. Faith Stone Miller and Dr. James A. Miller	"Some Effects of Strychnine on Reconstitution of Hydranth Primordia in Tubularia crocea."
Dr. Horace W. Stunkard	"The Life Cycle of Moniezia expansa."

Dr. Richard G. Abell and Dr. Eliot R. Clark	"A New Method for Studying the pH of the Intercellular Substance in the Living Mammal."
Dr. Richard G. Abell and Dr. Eliot R. Clark	"The Behavior of Living Mammalian Arterioles, Capillaries, and Venules When Exposed to CO_2."
Dr. Eliot R. Clark and Mrs. Eleanor Linton Clark	"The Control of Peripheral Circulation in the Mammal."
Dr. Louis Loeffler	"The Structure of the Liver Lobule."
Dr. John B. Gaylor and Dr. Ernst Scharrer	"A Preliminary Note on the Innervation of the Swim-bladder of the Sea-robin."
Dr. W. Gardner Lynn	"The Origin and Development of the Thyroid in Eleutherodactylus, an Anuran with No Tadpole Stage."
Miss Virginia Mayo	"Some Effects of Mammalian Follicle-stimulating and Luteinizing Hormones in Adult Female Urodeles."
Dr. G. H. Parker	"The Relation of Melanophore Responses to Vascular Disturbances."
Mr. E. P. Hiatt and Dr. J. K. W. Ferguson	"Some Effects of Chloroform on the Respiratory Systems of Yeast."
Dr. Jean Brachet	"The Oxygen Consumption of Activated and Fertilized Eggs of Chaetopterus."
Dr. M. E. Krahl, Miss Anna K. Keltch and Dr. G. H. A. Clowes	"Influence of Respiratory Inhibitors on Stimulation of Metabolism by Nitro and Halo Phenols."
Dr. G. H. A. Clowes, Dr. M. E. Krahl and Miss Anna K. Keltch	"Substituted Phenols as Inhibitants of the Fertilization of Arbacia and of Ciliary Movement of Arenicola Larvae."
Miss Anna K. Keltch, Dr. M. E. Krahl and Dr. G. H. A. Clowes	"Stimulation of the Rate of Cell Division of Arbacia Eggs by Carcinogenic Hydrocarbons."
Dr. Albert Tyler and Mr. N. H. Horowitz	"The Molecular Species Concerned in the Action of Substituted Phenols on Marine Eggs."

Dr. Kurt C. Stern and Dr. Kurt Salomon	"Ovoverdin, a Pigment Chemically Related to Visual Purple."
Mr. Louis-Paul Dugal and Dr. Laurence Irving	"The Increase of CO_2 and Decalcification in Certain Pelecypods."
Dr. J. K. W. Ferguson and Mr. E. C. Black	"The Effect of pH and Ionic Strength on the Activity of Carbonic Anhydrase."
Dr. J. K. W. Ferguson, Mr. S. M. Horvath and Dr. J. A. Pappenheimer	"The State of Carbon Dioxide in Dogfish Blood."
Dr. M. H. Jacobs and Dr. A. K. Parpart	"The Influence of Certain Alcohols on the Permeability of the Erythrocyte."
Dr. A. K. Parpart, Dr. M. H. Jacobs and Mr. A. J. Dzienmian	"Ionic Exchanges of Erythrocytes Inferred from Volume Changes."

PAPERS READ BY TITLE

Dr. L. G. Barth	"Oxygen as a Controlling Factor in the Regeneration of Tubularia."
Dr. Sinisha B. Bogdanovitch	"The Effects of Different Drugs on the Melanophores of Fundulus heteroclitus."
Dr. Sinisha B. Bogdanovitch	"Further Investigations on the Effect of Tissue on Different Drugs."
Mr. Barry Commoner	"A Quantitative Study of the Staining of Marine Eggs by Neutral Red."
Dr. John A. Frisch, S.J.	"The Adaptation of Paramecium to Sea-water."
Dr. Charlotte Haywood and Dr. Abby Turner	"The Water and Fat Content of Skeletal Muscle in Marine Fishes."
Dr. Walter N. Hess	"Reactions to Light of Different Intensities in Dolichoglossus kowalevskyi."
Dr. Hope Hibbard	"The Hatching of the Squid."
Dr. D. L. Hopkins	"The Vacuole System of the Marine Amoeba, Flabellula mira."
Miss Lois Hutchings	"Effect of Electrical Shocks upon the Division Rate of Stylonychia pustulata as Measured by the Interdivisional Period."

Dr. M. H. Jacobs and Mr. H. N. Glassman	"Further Comparative Studies on the Permeability of the Erythrocyte."
Dr. J. M. Johlin	"The Attenuation of Toxins by Interfacial Adsorption."
Miss Elsa M. Keil and Dr. F. J. M. Sichel	"The Action of Acetylcholine on the Skeletal Muscle Fibers of the Frog."
Mr. John A. Moore	"The Effect of Pituitary on Nuclear Changes in the Egg of the Frog."
Mr. Floyd Moser	"The Effect of Urea upon the Surface of Unfertilized Arbacia punctulata Eggs."
Mr. Floyd Moser	"The Cortical Response of Arbacia punctulata Eggs to Direct Current."
Dr. Magnus Olson	"The Histology of the Retractor Muscles of Thyone briareus Lesueur."
Dr. C. Ladd Prosser and Mr. A. H. Chambers, Jr.	"Strength-Duration Curves of Nerve Fibers in the Squid."
Dr. Leonard P. Sayles	"New Structures Induced by Implants of Adult Nerve Cord in the Polychaete, Clymenella torquata."
Dr. A. A. Schaeffer	"Cytoplasmic Division in Type B of the Giant Amoeba Chaos chaos Linnaeus."
Dr. Ernst Scharrer	"The Sense of Taste in the Free Fin Rays of the Sea Robin (Prionotus)."
Dr. Victor Schechter	"Calcium and Magnesium in Relation to Longevity of Mactra, Nereis and Hydroides Egg Cells."
Dr. F. O. Schmitt and Dr. Otto H. Schmitt	"A Convenient Method for the Measurement of Nerve Respiration."
Dr. F. J. M. Sichel and Dr. C. Ladd Prosser	"Temporal Relations in the Excitation of the Isolated Muscle Fiber."
Dr. H. Burr Steinbach	"Electrolytes in Phascolosoma Muscle."
Dr. Harry N. Stoudt	"Leaf Development and Vegetative Propagation in Polystichum plaschnickianum."
Dr. T. Terni	"Morphological and Experimental Cytology of Lobster Spermatozoa."
Dr. Abby H. Turner and Dr. Charlotte Haywood	"Observations on Arterial Pressure in Marine Fishes."

Dr. Albert Tyler, Miss Nelda Ricci and Mr. N. H. Horowitz	"Respiratory Rate and Length of Fertilizable Life of Unfertilized Arbacia Eggs under Sterile and Non-sterile Conditions."
Dr. A. J. Waterman	"The Effect upon Gastrulation and Differentiation in Arbacia of $NiCl_2$, $CuCl_2$, and Na_2SiO_3 in Modified Artificial Sea Water."
Dr. Ralph Wichterman	"Studies on Living Conjugants of Paramecium caudatum."
Dr. Ralph Wichterman	"Conjugation in Paramecium trichium Stokes (Protozoa, Ciliata) with Special Reference to the Nuclear Phenomena."
Dr. Opal M. Wolf	"Mitotic Activity of Stimulated Rat Adrenals Measured by Colchicine Technique."
Dr. Opal M. Wolf	"Induced Breeding Reactions in Isolated Male Frogs, Rana pipiens Schreber."

DEMONSTRATIONS

Thursday, August 26

Dr. S. E. Pond and Mr. L. F. Boss	"Sources of Working Current for a Potentiometer."
Dr. R. Chambers and R. B. W. Zweifach	"Micro-manipulative Studies of Blood Capillaries."
Dr. J. E. Kindred	"Stages in the Development of Lymph Nodules."
Dr. E. A. Wolf	"Individual Variations in Salivary Amylase in Man."
Dr. K. S. Cole, Dr. H. J. Curtis and Mr. J. M. Spencer	"Alternating Current Wheatstone Bridge for Impedance Measurements at Frequencies from 30 to 10,000,000 Cycles."
Dr. E. R. Clark and Mrs. Eleanor L. Clark	"Control of the Peripheral Circulation as Seen in the Living Mammal."
Dr. R. G. Abell and Dr. E. R. Clark	"A New Method for Studying the pH of the Intercellular Substance in the Living Mammal."
Dr. V. Hamburger	"Limb-bud Transplantation in Chick Embryos."

Mr. J. A. Smith	"Types of Hermaphroditism in Mollusca."
Dr. F. O. Schmitt and Dr. O. H. Schmitt	"A Convenient Method for the Measurement of Nerve Respiration."
Dr. F. O. Schmitt, Dr. O. H. Schmitt and Dr. R. S. Bear	"Action Potentials from the Giant Axon of the Squid, Loligo pealii."
Dr. S. E. Hill	"Action Currents in Nitella."
Dr. T. M. Sonneborn	"Sexuality in Paramecium."
Dr. J. B. Gaylor	"Innervation of the Swim-bladder of the Sea-robin."
Dr. E. Fischer	"Volume Changes of Muscle on Contraction."
Dr. C. E. McClung	"Relation of the Apical Cell to the Germ Cell in the Grasshopper Testis."
Dr. H. Irene Corey	"Polar Elements of Orthopteran Chromosomes."
Dr. D. H. Wenrich	"Mitosis in Dientamoeba fragilis."
Dr. C. L. Parmenter	(a) "Haploid, Diploid, and Triploid Mitoses in Parthenogenetic Frog Tadpoles." (b) "Mono, Di, Tri, and Tetra-nucleate Blood Cells in Haploid Parthenogenetic Frog Tadpoles."
Dr. A. A. Schaeffer	"Types A and B of Chaos chaos Linnaeus, the Large Multinucleate Amoeba of Roesel."
Dr. A. K. Parpart	(a) "Method for the Rapid and Complete Separation of Erythrocytes from a Surrounding Medium." (b) "Volume Changes of Erythrocytes Produced by an Ionic Exchange."
Dr. G. Failla	"New X-ray Equipment of the Marine Biological Laboratory."
Dr. T. Terni	(a) "Morphological and Experimental Cytology of Lobster Spermatozoa." (b) "The Nervous System of Dogfish Embryos."
Mr. L. F. Boss and Dr. S. E. Pond	"Combination Thermostat, Heat Interchanger and Relay Unit for Constant Temperature Bath."
Mr. C. G. Grand	"Cytolytic Effects on Marine Eggs after Puncturing with Microneedles."

Dr. M. J. Kopac	(a) "Living Valonia Aplanospores." (b) "Apparatus for Delivering Oil Drops under Constant Pressure."
Dr. Eleanor Carothers	(a) "Development of Mid-gut of Melanoplus differentialis." (b) "Effects of X-rays on Grasshopper Embryos."
Dr. A. Hollaender	"Equipment for Work with Measured Quantities of Monochromatic Radiation in the Visible and Ultraviolet."
Mr. L. F. Boss, Dr. A. Hollaender and Dr. S. E. Pond	"Inexpensive 500 Volt D.C. Power-supply for High Intensity Ultraviolet."
Dr. B. R. Nebel and Dr. E. B. Harvey	"Cytological Observations on Arbacia Eggs Activated by Monochromatic Ultra-violet Radiation."
Dr. L. Loeffler	"Injected Liver of Toadfish."
Dr. R. W. Root and Dr. W. Etkin	"Use of the Constant Flow Method for Serial Determinations of Oxygen Consumption in Toadfish."

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THE REPRODUCTIVE CYCLE OF BRACHYRAPHIS EPISCOPI, AN OVOVIVIPAROUS PŒCILIID FISH, IN THE NATURAL TROPICAL HABITAT

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INTRODUCTION

Reproduction in pœciliid fishes occurs in short recurring cycles. Field studies on the reproduction of these fishes, principally *Gambusia affinis* and *Heterandria formosa*, in the temperate and subtropical zones, have demonstrated that reproduction reaches its peak in spring and early summer, declines during late summer and fall, ceases altogether during the winter and begins again in early spring. Laboratory studies have shown that reproduction is continued longer in the fall and begins earlier in the spring when the gravid females are kept under constant conditions as regards temperature and food. The decline in the reproductive period would be associated with other factors, either genetic or some uncontrolled environmental factor. In a laboratory study of various pœciliids, principally *Xiphophorus helleri*, *Lebistes reticulatus*, *Pœcilistes pleurospilus* and *Heterandria formosa*, the writer (Turner, 1937) came to the conclusion that the seasonal variation of light was a factor in controlling the reproductive cycle, probably through the action of the pituitary. Hoover and Hubbard (1937) have produced experimental evidence that exposing maturing brook trout to periods of light in excess of those of seasonal daylight will induce early maturing and spawning.

In order to test the possibility of light as one of the controlling factors in the reproductive cycles in pœciliids, one might artificially control the light exposure so that it would be uniform throughout the year and equal daily to that under which the fish would reproduce freely. Or, a study might be made of the reproduction of a pœciliid fish under natural tropical conditions in which the daily light exposure is fairly uniform and in a selected habitat in which food and temperature change but little during the annual period.

This paper is written to record the results of a study of reproduction in *Brachyrhaphis episcopi*, a pœciliid fish which is common in a small

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[1] This investigation was supported by a grant-in-aid from the Graduate School of Northwestern University. The writer is greatly indebted to Mr. James Zetek, curator of the Barro Colorado Island Laboratory, for his coöperation and help.

stream near the laboratory at Barro Colorado Island in the Panama Canal Zone about nine degrees north of the equator. The fish was chosen because it is a typical pœciliid, was easily accessible, and occurred in sufficient numbers during all seasons of the year to make a critical study feasible. Collections were made during late June and early July in the summer of 1936 with a wide silk bolting cloth dip net in such a way as to secure a fair sample of all sizes of *Brachyrhaphis* in the stream. An employee of the laboratory was instructed in the manner of collecting and through the kindness of Mr. James Zetek, the collections were shipped to the writer. The specimens were fixed in formalin and transferred to 70 per cent alcohol. Collections representing all sizes obtainable were obtained for each month except May.

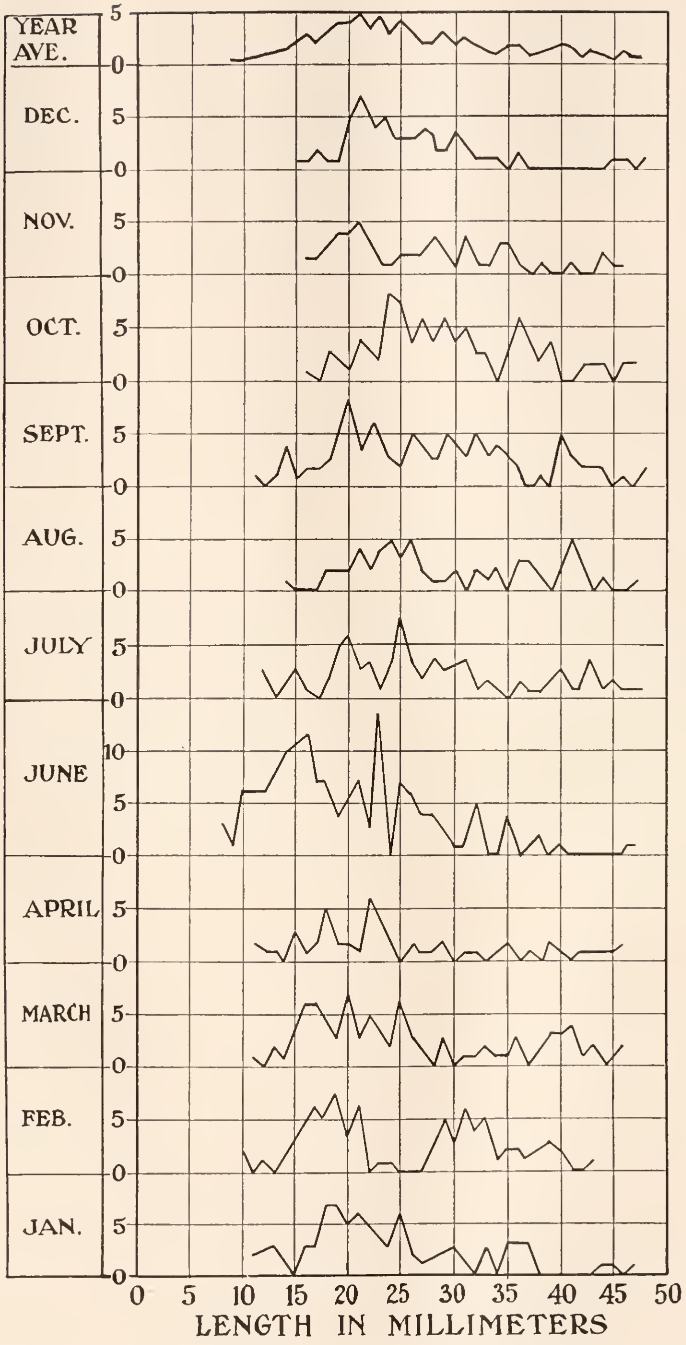
In all, 1,139 immature and female fishes were collected and measured and 442 were dissected for the purpose of counting and staging the embryos and measuring the developing ovocytes. A total of 288 mature or partially mature males were examined.

Allee (1926) made a careful study of the variations in temperature, rainfall and other environmental features at this locality and reported that the seasonal variation in temperature on the jungle floor through which this stream flows is slight. During the rainy season there is an abundance of water in the stream but during the dry season the stream is reduced to a series of connected pools. At no time is the water in the pools more than two feet deep. Floating debris, insect adults and larvæ and other small animals upon which the fish feeds are abundant. The daily duration of daylight varies from eleven hours and thirty-six minutes in late December to twelve hours and forty minutes in late June. This natural habitat in general has little variation in food supply, temperature and light.

Lengths of Fishes in Monthly Collections

Some information may be obtained by taking the lengths of all the fishes obtained in each month and comparing them throughout the year. Such a record is shown in Fig. 1. If a seasonal breeding cycle exists it might be expected that the minimal size would occur at the beginning of the breeding season and that the minimal size would increase as growth takes place. If the results are plotted graphically it might be expected also that a mode for the entire population would gradually move to the right as all fishes continued to grow.

It will be noted that the point for the minimal length, representing newly born fishes, does not move to the right. Specimens between 8 and 17 mm. in length are present in each month. It is believed that the minimal size represented in June is more characteristic than that



Text Fig. 1. Graph showing relative numbers of fishes of each length for each month. Numbers in each month are shown vertically, lengths of specimens in millimeters horizontally.

obtained for other months. The writer made the June collection and took great pains to secure the smallest fishes in the stream. The other collections were made by the caretaker at the laboratory and it is likely that the smallest specimens were overlooked, especially since they have a tendency to hide in the vegetation at the stream margin. The evidence indicates that young are being produced in each month of the year.

If a graph is plotted based upon the entire annual collection instead of the monthly collections, the mode for the entire population will appear in lengths between 20 and 30 mm. When the monthly curves are smoothed it will appear that the largest number occurs in each month at the same length. Since the mode shows no tendency to move to the right during any season it is assumed that it is maintained in its position by a continual new supply of young.

As far as this evidence goes it indicates that there is no season relation in the production of young and that the fall decline and winter cessation in reproduction found in the pœciliids kept in laboratories at a latitude of about forty degrees are lacking.

Brood Size in Different Months and Seasonal Occurrence of Gravid Females

If reproduction is seasonal it would be expected that during some months gravid females would be lacking altogether or that a smaller proportion of females would be carrying broods. Except in the smallest of the mature females no such relation was found. Gravid females occurred in each month and in about the same proportion. An occasional female from 24 to 26 mm. in length is found to be carrying young but the data are too scanty for any conclusion concerning a seasonal relation to time of maturity.

In some pœciliids studied in the laboratory (Turner, 1937) not only does the interval between broods become shorter as reproduction approaches its height but there is an increase in the number of embryos in succeeding broods. With this in mind, all the gravid females of *Brachyrhaphis* obtained in each month were dissected to determine whether the number of embryos in each brood varied seasonally. Monthly averages of brood size for each length of gravid female are indicated in Table I. The data show that: (1) There is considerable variation in brood size for each length of gravid female. (2) There is no seasonal trend for size of broods in gravid females of any length.

The conclusion from this evidence indicates again that reproduction is continual throughout the year and that no seasonal increase or decrease in reproductive activity exists.

Incidental information is obtained concerning the fecundity of females of different lengths and of the relative contribution to the population of females of different lengths by considering brood size for each length and the relative abundance in the population as a whole of females of different lengths (Table III). It will be noted that brood size is very small in 25 to 30-mm. females and that it rises to a maximum in the largest females. The total production of young by the largest females is not greater, however, than that of females somewhat smaller because of their relatively smaller numbers in the population as a whole.

Table I

Table indicating brood size in gravid females of different lengths for each month

Length of gravid female in mm.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Yearly average for each length
24													
25	1												1
26	2.5			3									2.9
27	4	2	3									4	3.25
28	6			1						3		3	3.25
29	9	1	1.5	1					2			4.6	3.18
30	6		8	6.5					3		5	3	5.25
31	6			3		6		4	3	3	4	5.5	4.3
32	7					4				4	5		5
33	10	7		5		7	7			5	6.5		6.9
34		7	7			11	10					6.5	8.3
35	8.6			8		8	9				5.5		8.4
36	17.5	9	5.5			9	9	5		3		14	9
37	6		5			5.5	9.5	6		7			6.5
38			8	8.6		19.5	18.5	8.5	3	8			10.6
39			11.5	12		12.6	9.7		5.5	10.5			10.3
40		8	8	9			10	6	12.5				8.9
41		8		11			11.5	9.5	12		4		9.6
42			13.5	18			28	9	5		19		15.4
43						25.5	14	18	11	10		30	19.1
44		30	14				13	15	8	18.5	22		17.2
45	28	41					18	15		13	23	9	21
46			23			35				20		20	23.6
47	46	30	17					21	29	24			27.8
48									13			40	26.5
Average for all lengths per month	11.3	14.3	9.6	7.2		12.7	12.2	10.3	9.2	9.9	10.4	8.9	

Relation of Ovocyte Development to Embryo Stages

In a study of the relation of ovocyte development in gravid ovaries to stages of embryos in the same ovaries (Turner, 1937), it was discovered that different relationships exist in different groups of pœciliids. In *Gambusia* the ovocytes of a gravid ovary remain small until the brood of embryos in the ovary is evacuated. In *Xiphophorus*, *Lebistes* and some other species the ovocytes increase in size while the ovary is gravid and in *Quintana atrizona* the ovocytes have grown to full size and are ready for fertilization immediately after the evacuation of a brood. In *Pœcilistes pleurospilus*, *Pœciliopsis infans* and *Heterandria formosa*, the ovocytes have grown not only to maximal size but fertilization has taken place before the older brood of embryos is evacuated and two or more broods of embryos are found in the ovary at the same time. The condition described above occurs in laboratory-reared specimens at the height of the reproductive season. When reproduction is at a low ebb the ovocytes grow slowly and are relatively much smaller at the time of the birth of the embryos than they are when reproduction is at its height.

Table II

Table showing stages of developing ovocytes for each stage of developing embryo in the same gravid ovary for each month

Stages of embryos in gravid ovaries	Developing ovocytes: diameter in mm.												
	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Average
Stage 0.	.38	.28	.23	.21		.26	.28	.34	.29	.19	.28	.20	.27
Stage No. 1		.3				.25	.2					.22	.24
Stage No. 2	.27		.13	.25		.21	.2		.24	.15	.25	.26	.22
Stage No. 3	.46	.25	.20	.25		.29	.29	.22	.23	.21	.24	.24	.26
Stage No. 4	.50	.56	.27	.64		.72	.51	.35	.55	.33	1.05	.64	.55
Stage No. 5	1.28	1.05	.86	1.41		.90	1.3	.98	1.40	1.03	1.39	1.37	1.18

This latter fact is used as a third method of searching the material for evidence of a seasonal variation in reproduction. If a seasonal high and low phase of reproduction exists the growing ovocytes should be smaller at the low phase and larger at the high phase provided that they were referred to some fixed stage in reproduction such as the birth of the embryos or to some definite stage of development in the embryos of a brood. Gravid females were dissected and the embryos in the ovary were staged (Table II). Stage 0 represents a condition in the ovary in which no embryos are present and the ova which are to form the next brood are full-sized but not yet fertilized. Stage 5 represents a condition in which the embryos are ready for birth and stages 1, 2, 3 and 4 represent conditions in which the embryos are in intermediate stages. At any stage except 0 there is a group of developing ovocytes definitely advanced over younger stages and about equal in number to the embryos in the brood held in the same ovary. These developing ovocytes vary somewhat in diameter so that it was necessary to take an average diameter.

The findings are shown in tabular form in Table II. A monthly average of the diameters of developing ovocytes is shown for each embryo stage in the broods held in the ovaries. A yearly average is also shown. In general it will be noted that the ovocytes do not increase in size during stages 0, 1, 2 and 3 but increase over four-fold during stages 4 and 5. A comparison of the monthly averages with the yearly averages indicates that there is considerable variation, probably due to the small numbers of specimens examined, in the diameter of the ovocytes from month to month, but that no definite trend is shown toward a seasonal increase or decrease.

TABLE III

Table indicating productivity of females of different lengths

Length in mm.	No. of gravid females; yearly average for each length	Average no. of young per gravid ovary	Relative productivity
25	4	1	4
26	3.2	2.9	7.9
27	2.4	3.25	7.8
28	2.5	3.25	8.17
29	3.2	3.18	10.17
30	2.3	5.25	12.07
31	2.6	4.3	11.18
32	2.2	5	11
33	2	6.9	13.8
34	1.2	8.3	9.9
35	2	8.4	16.8
36	2	9	18
37	1.2	6.5	7.8
38	1.3	10.6	13.9
39	1.5	10.3	15.5
40	1.7	8.9	15.1
41	1.5	9.6	14.4
42	1	15.4	15.4
43	1.2	19.1	23.0
44	1	17.2	17.2
45	.6	21	12.6
46	1	23.6	23.6
47	.6	27.8	16.6
48	.5	26.5	13.2

Age of Maturity in Males

In the males of pœciliid fishes maturity is marked externally by the development of a gonopod, formed by a metamorphosis of the anal fin. The testis develops and spermatogenesis begins at the same time or a little earlier. Once maturity has been attained the production of spermatozoa seems to be continual. Males will reproduce during any season in the laboratory and attempts to find seasonal waves either in spermatogenesis or in reproductive behavior have failed. Any study of the testis in *Brachyrhaphis* for evidence for a seasonal variation in maturation stages in adults would not be expected to yield results and no such study was attempted.

A study was made of the size at which maturity is indicated by the development of the gonopod and of the relative numbers from month to month of the mature males and of males with partially metamorphosed anal fins. The results are summarized as follows: (1) Some males mature at an early stage and have fully metamorphosed gonopods when they are 15 mm. in length. (2) Some males postpone maturity and specimens with partially metamorphosed gonopods may be found at any length up to 36 mm. Specimens with transforming gonopods are most abundant at lengths from 16 to 25 mm. (3) Specimens of 15 mm. with gonopods fully developed and larger specimens with developing gonopods were found in each month in which material was obtained.

These findings indicate that there is no seasonal variation in the production of maturity in males.

Discussion and Summary

Three lines of approach are used in the study of females and immature specimens to determine whether there exists in *Brachyrhaphis episcopi*, living in a natural tropical habitat, a seasonal variation in reproduction such as exists in pœciliid fishes living in natural habitats in sub-tropical and temperate zones and in laboratory-reared specimens maintained in the temperate zone. It is concluded that reproduction occurs in the short cycles characteristic of pœciliid fishes but that the cycles are continuous and are not seasonal for the following reasons: (1) Young are produced at about the same rate in each month of the year as indicated by measurements of lengths of all specimens taken in each month. (2) Gravid females are taken in each month, indicating that fertilization takes place the year round. (3) The relative proportion of gravid to non-gravid females in each month is approximately equal. (4) The age, as indicated by length of specimens at which females become mature, shows no significant seasonal variation.

(5) The average number of embryos in a brood for each size of female shows little seasonal variation. (6) In the same gravid ovary the relative interval between embryos in definite stages and the stage of development of developing ovocytes does not vary seasonally.

A study of the age of maturity of males and of the relative abundance of mature and partially mature males during the different seasons indicates also that no seasonal variation exists.

In temperate and sub-tropical zones in natural habitats the recurring reproductive cycles in pœciliid fishes decline in late summer and fall and cease altogether in the winter. Here temperature is obviously one factor which is related to reproduction. It has been shown, however (Barney and Anson, 1921*b*), that a decline in reproduction begins while the temperature of the water is still favorable for further reproduction. In pœciliids maintained in the laboratory with food and temperature controlled but with the exposure to light varying with the seasonal fluctuation in daylight, reproduction rises with the approach of spring and wanes during late summer. It is assumed that light is also a factor in the control of reproduction and that the rather uniform seasonal duration of daylight at the latitude of the Panama Canal Zone (9° N.) is concerned in the maintenance of a continual reproduction in *Brachyrhaphis*. The minimal duration of daylight here at 9° N. is eleven hours and thirty-six minutes and is equivalent to the duration of light on March 7 at a latitude of 42° N (Chicago latitude). The maximal duration at 9° N. is twelve hours and thirty-nine minutes and is equivalent to that on April 1 at a latitude of 42° N. If duration of daylight is concerned as a factor partially controlling reproduction, it must be concluded that for *Brachyrhaphis* at least the excess of daylight in late spring and early summer at 42° N. latitude would be unnecessary for the maintenance of reproduction and the question naturally arises as to the effect of this excess of daylight on the reproduction of fishes which occur in natural habitats at 42° N. latitude. It will be interesting to determine whether pœciliids other than *Brachyrhaphis* will reproduce continually when placed under experimental conditions with food and temperature controlled and with exposure to light varying within the limits of duration and intensity occurring at 9° N. latitude.

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THE SEASONAL CYCLE IN THE GONADS OF FUNDULUS

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From the voluminous work on mammals, birds, and amphibians and to a less extent on reptiles also, the factors concerned in the control of the sex cycle in these forms are becoming more clearly defined. Fishes, however, have received little attention in this respect. Experiments designed to investigate such factors as might be involved in controlling the annual sex cycle which *Fundulus heteroclitus* exhibits were begun some time ago. When an attempt was made to analyze the results of these experiments it was clear at once that before any orderly arrangement of data could be made it would be necessary to determine the condition of the gonads at intervals during the normal seasonal cycle. Accordingly normal individuals were collected at regular intervals over a period of fifteen months, for the most part from the Delaware River near Palmyra, New Jersey, though animals were obtained at Woods Hole during June, July and August. These animals were killed either immediately or within a week after collection, the animals and gonads were weighed, and the gonads, pituitary and thyroid glands were preserved for sectioning. The following is a report on the seasonal variation in the gonads of these animals.

The male *Fundulus* is provided with a pair of testes, elongate organs lying one on either side of the mid-line, closely pressed against the ventral wall of the swim bladder to which they are attached by short mesorchia (Fig. 1). In general the two testes are separated for the greater part of their length by a mesentery which supports the digestive tract, but they may be joined to an extent which varies in different individuals. A short duct leaves the posterior end of each testis, joins the corresponding duct from the opposite side and runs to the genital pore which, in the male, is at the tip of a short, conical elevation at the base of the anal fin.

Each testis has a thin fibrous capsule which is provided with a mesothelial covering. Beneath the capsule a central medulla and a peripheral cortex can be distinguished (Fig. 2). The medulla is made up of fibrous connective tissue in which are embedded many sperm ducts, blood vessels and clusters of peculiar cells which will be described below. The cortex is divided into somewhat indistinct lobules by small amounts of connective tissue, each lobule containing several cysts which are closely packed with spermatogenic cells. In these sections, which were not prepared for special cytological details, only four types of spermatogenic cells could be distinguished. The largest of these, cells with large nuclei and one or two prominent nucleoli, are similar to the primary spermatogonia described by Turner (1919) in the perch and by Foley (1926) in *Umbra*. The next largest cell, about two-thirds the size of the primary spermatogonia, is one in which the nucleus occupies a much larger percentage of the cytoplasm. These are the most actively dividing cells of the testis. They must include secondary spermatogonia as well as both primary and secondary spermatocytes but these three cell types could not be distinguished with accuracy. The third kind of cell observed is somewhat smaller with a deep staining nucleus and is undoubtedly the spermatid. Finally spermatozoa with their rounded heads and elongate tails can be readily distinguished. In any one cyst all the cells are alike.

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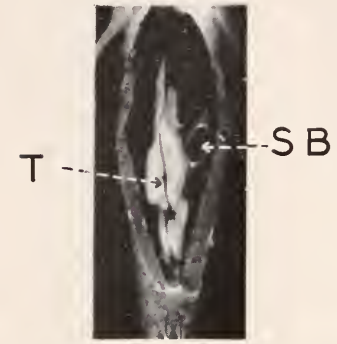
[1] The early work on this material was carried out at the Department of Anatomy, University of Pennsylvania.

The seasonal cycle observed in the testes of these animals is as follows. In late May, June, and July the medulla is swollen with ducts filled with sperm (Fig. 3). Nearly all the cysts of the cortex are also filled with sperm but around the periphery there is a narrow zone composed mainly of primary spermatogonia. These cells are dividing rapidly, producing small cysts of secondary spermatogonia. Thus before the matured sperm of one generation of sex cells is completely expelled the beginnings of the next generation are apparent. Ordinarily the sperm are shed during June and July. In some cases, however, the sperm are not expelled until the last of August. Complete expulsion of the sperm is rare. Small clusters of sperm which are apparently left from the previous period of sexual activity can be found in nearly every testis examined regardless of the time of year. After the spawning season there is a striking reduction in the amount of cortical tissue relative to the total diameter of the testis, even though the preliminary stages in the production of the next generation of sperm have already produced small cysts (Fig. 4). Cells within these cysts continue to divide slowly, the cysts gradually enlarge, and the cortex of the entire testis increases in thickness. In September a few spermatids appear. Throughout the period from October to the latter part of February there is a gradual increase in the number of spermatogonia, spermatocytes, and spermatids, and the cysts become larger. Sometime in March the cells within the cysts begin to divide more rapidly, as evidenced by the number of mitotic figures and the rapid increase in size of the cysts during this period, and spermatids become increasingly more numerous in proportion to other cells. In late April and in May spermatozoa appear in increasingly large numbers.

During this process the testis changes markedly in color and size. The active testis found during May, June and the early part of July is



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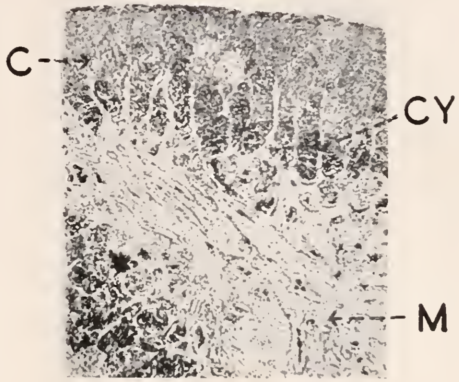

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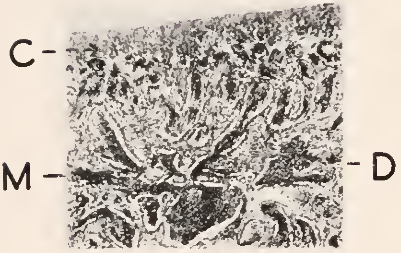

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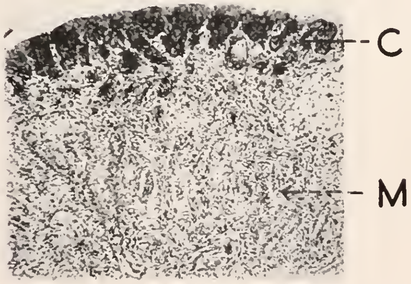

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Plate I

Fig. 1. Male *Fundulus*, digestive organs removed. × 1.5. *SB*, artificial opening in ventral wall of swim-bladder to show its relation to the testis; *T*, testis.

Fig. 2. Cross-section of testis of animal killed 12/4/35. × 80. *C*, cortex; *CY*, cysts of spermatogenic cells; *M*, medulla.

Fig. 3. Cross-section of "ripe" testis of animal killed 7/30/35. × 80. *C*, cortex; *D*, sperm duct filled with sperm; *M*, medulla.

Fig. 4. Cross-section of exhausted testis of animal killed 7/30/35. × 80. *C*, cortex; *M*, medulla.

milky white and definitely enlarged. The exhausted testis in late July and August is colorless for the most part, more or less transparent and much reduced in size. Figure 5 indicates the seasonal changes in weight of the testis relative to the total body weight. This chart is based on data from 58 animals collected over a period of fifteen months, from December 1935 to March 1937 inclusive, the average weights for the calendar months being taken. While the number of animals on which this chart is based is insufficient for accurate statistical analysis it is apparent that the testis forms the greatest percentage of the total body weight during the period from late in March to the latter part of June. When the averages for the months of January, February and March, 1937, are separated from those of the same months of the preceding year and the curve is drawn for a period of fifteen months the differences between such a curve and the one shown are slight and are probably due to the small number of animals which could be collected during this period of the two years. Such a curve also shows a rise in weight of the testis during March of both years. Sections of the testes of these animals show that the greatest weight of the testis corresponds to that period in the seasonal cycle from the beginning of rapid division of spermatogenic cells and the consequent enlargement of the cysts that occurs in March up to the time when the majority of the sperm is expelled, usually in June or July. This curve for the proportion of the body weight formed by the testis in *Fundulus* at different periods throughout the year is somewhat different from a similar curve for the perch (Turner, 1919). Although in this latter form the spawning season is only a month or so earlier than that in *Fundulus*, the sudden increase in weight of the perch testis appears at the end of the summer, notably earlier than it occurs in *Fundulus*.

In the medulla of the testis clusters of peculiar cells which might be considered interstitial cells were found. Much has been written concerning the nature, origin, and function of interstitial tissue in teleosts. They are apparently absent in many teleosts (Kolmer and Scheminzky, 1922), present in others (Stieve, 1933). Courrier (1921) believes they arise from leucocytes that migrate into the testis, and that they are responsible for the development of such secondary sex characters as the color patterns in males. Van Oordt (1925), on the other hand, thinks it is improbable that there is in the fish testis a group of cells which are a specific source for sex hormones. In *Fundulus*, after the sperm are shed late in July, there are present in the medulla of the testis numerous clusters of cells with shrunken, pycnotic nuclei. The cytoplasm of these cells stains pale yellow and frequently has a large vacuole (Fig. 6). These cell clusters are most conspicuous just after the sperm are shed but they persist for long periods and can be found in all the testes examined except in those fixed either immediately before or during the spawning season, when the connective tissue in the medullary region is reduced to narrow strands by the large number of spermatogenic cells present. The origin of these cells is doubtful. In many cases during late April, May and June, when the testis is well filled with spermatogenic cells and the ducts are enlarged and filled with sperm, between these ducts cells can be found with a larger amount of cytoplasm than is present in other cells of the connective tissue. Some of these are pale yellow, others light pink in the sections, and some have vacuoles which may be empty or may contain a pink-staining homogeneous substance. Their position and appearance suggest that it is these cells which develop into the shrunken, vacuolated, pale yellow cells found in testes fixed after the sperm have been shed. They are the only cells found which might be called interstitial cells. There is no evidence, however, that they are secretory. Certainly the shrunken cells found after the sperm are shed resemble atrophic rather than active cells.

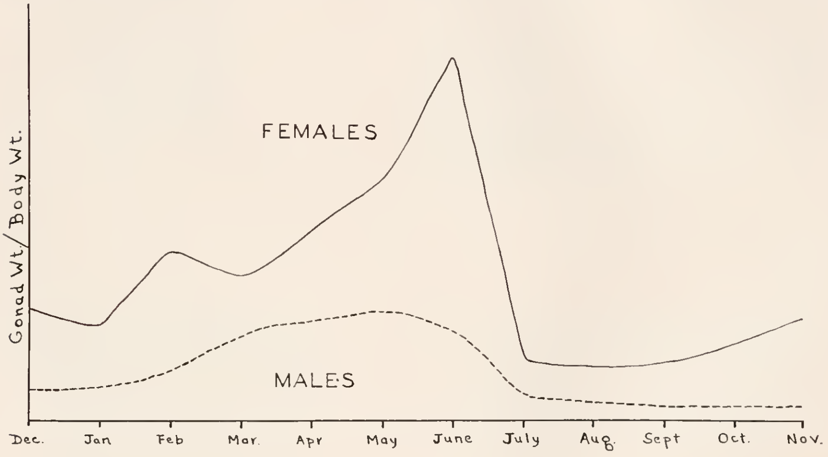

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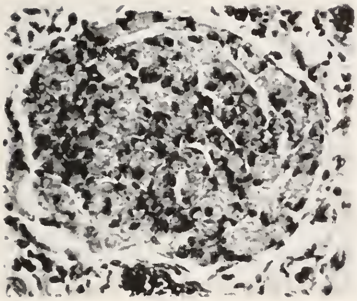
FIG. 5. Seasonal variation in gonad weight, expressed as percentage of body weight. Dotted line: testis; solid line: ovary.

The ovary of *Fundulus* is unpaired but is bi-lobed at its anterior end and has a mid-ventral groove that separates it superficially into right and left portions. It occupies the same position in relation to other organs of the body cavity as does the testis in the male. It is covered externally with a layer of peritoneum beneath which is a thin tunica albuginea of connective tissue in which may be found smooth muscle



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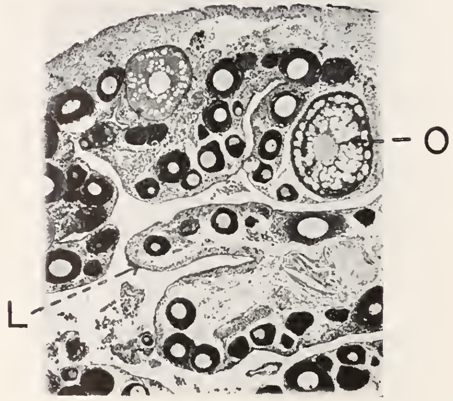

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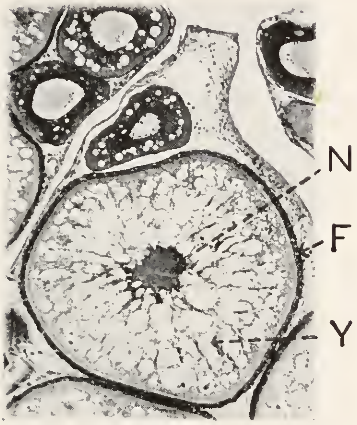

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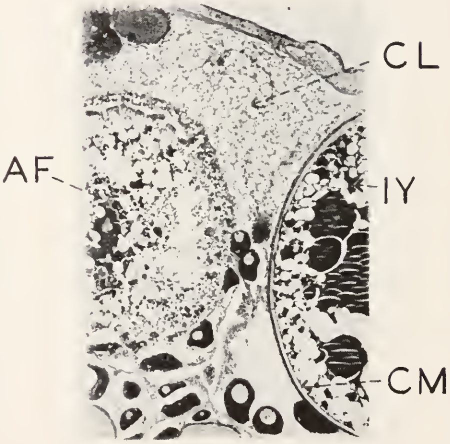

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PLATE II

FIG. 6. Interstitial cells of testis. × 340.

FIG. 7. Cross-section of ovary of animal killed 9/22/36. × 47. *O*, oöcyte showing vesicular yolk; *L*, ovigerous lamella.

FIG. 8. Oöcyte from animal killed 3/16/37. × 80. *F*, follicle; *N*, nucleus with several peripheral nucleoli; *Y*, vesicular yolk.

FIG. 9. Ovary of animal killed 6/11/36. × 47. *AF*, atretic follicle with secondary yolk present; *CM*, chorionic membrane of nearly ripe follicle; *CL*, "corpus luteum"; *IY*, secondary yolk.

fibers (Fig. 7). These smooth muscle fibers are most conspicuous in ovaries containing ripe or nearly ripe eggs or in those from which the eggs have been recently shed. Within the tunica albuginea are many ovigerous lamellæ which project into the lumen of the ovary. These folds are covered with an epithelial lining continuous with that of the oviduct, a single short structure leading from the lumen of the ovary to the genital opening posterior to the anus. The condition in the female differs from that in the male in that the external genital opening is found at the tip of a fold of skin which carries the opening a short distance posteriorly along the anterior ventral border of the anal fin.

Sections of the ovary made after the spawning period show that all mature oöcytes have either been shed or have become atretic. In these ovaries there can be found, just beneath the epithelium covering the ovigerous lamellæ, numbers of small cells with large, vesicular nuclei, each with a prominent nucleolus. Practically no cytoplasm is visible. While these cells may be found at any period of the year, the large number of mitotic figures found in them after the spawning season indicates that this is a period of active proliferation. In subsequent development both nucleus and cytoplasm enlarge, the latter the more rapidly, and the number of nucleoli increases from one to several. The cytoplasm is deeply basophilic. In later stages yolk vesicles appear (Fig. 7), few at first but increasing in number until the oöcytes are completely filled with these save for a peripheral ring of granular cytoplasm. By the time this condition has been reached, the nucleus presents numerous nucleoli which are arranged in a ring just beneath the nuclear membrane (Fig. 8). The wall of the follicle, which at first consisted of only a few flattened cells, is now composed of a large number of cuboidal cells beneath which the chorionic membrane makes its appearance. In subsequent development there appears within the primary yolk vesicles what Guthrie (Marza, Marza, and Guthrie, 1937) calls intravesicular yolk (Fig. 9). While the contents of the primary vesicle are dissolved out in preparing the sections, this secondary yolk appears in sections as a homogeneous mass which stains faintly reddish with eosin. This intravesicular yolk increases in amount and at the same time yolk of the same sort appears between the vesicles and also increases in amount. The fully ripened oöcyte is completely filled with yolk except for the nucleus, located at one pole, and a few peripheral vacuoles. The chorionic membrane becomes thick and brittle. After the egg is discharged from the follicle the follicular epithelium undergoes marked hypertrophy, the cells developing large amounts of some substance which is dissolved out during preparation of the sections, leaving large vacuoles in the cells which thus resemble fat (Fig. 9). Mitotic figures have not been found in these follicular cells during the formation of this structure. This "corpus luteum," which is actually yellow in color in the fresh condition and can be seen on the surface of the ovary as a yellow spot, gradually shrinks and disappears, though traces of it may be found as late as December or January.

During this process of maturation of the oöcytes some of the follicles undergo atresia. This is particularly noticeable when the spawning season approaches and the ovary is greatly distended by the growth of many follicles. The epithelium of the atretic follicle, provided atresia occurs late in development, may undergo changes similar to those described for the epithelium of the ruptured follicle, though not as marked in degree. The smaller size of the atretic follicles as well as the remains of the oöcytes within them serve to distinguish them from the corpora lutea developed in follicles from which eggs were shed (Fig. 9).

Certain seasonal changes in the ovary may be noted. At the end of the spawning season, late in July, all ripe eggs have been expelled and there is found in the ovary corpora lutea and atretic follicles as well as all stages in the development of oöcytes from the initial stage up to and including the late development of vesicular yolk. As has already been mentioned, the smallest recognizable stage of the developing oöcyte can be found just within the epithelium lining the ovigerous lamellæ at any time throughout the year, so it is conceivable that these begin maturation at different times during the year. Hence it is not clear whether these larger oöcytes found at the end of the spawning period belong to the same generation of cells as those which matured completely and were shed, or whether they are maturing oöcytes which began development somewhat later.

Changes in gross appearance and in weight of the ovary are more striking than similar changes in the testis. The "ripe" ovary is recognizable at a glance by the presence of numerous ripe eggs which appear as large, pale, semi-transparent spheres that cause the wall of the ovary to bulge at numerous points. The exhausted ovary, on the other hand, is very much reduced in size and the largest oöcytes which it contains are much smaller than ripe eggs and are yellowish and opaque. The weight changes are indicated in Fig. 5, comprising the data from 98 females. Sections of these ovaries show clearly the relation between the weight curve and the structure of the ovary. Just after the spawning period the ovary comprises the smallest percentage of the total body weight. From this point on there is a gradual increase from month to month in the weight of the ovary, paralleled by an increase in size of the follicles and their contained oöcytes and in the number of yolk vesicles. The chorionic membrane appears as early as October in a few oöcytes, the number of chorionic membranes and the amount of yolk increasing during subsequent months. Droplets of intra-vesicular yolk may appear as early as March or even February, but rapid development of this secondary yolk takes place late in April, increasing rapidly in amount from then up to the time of spawning. The maximum percentage of the body weight formed by the ovary is found in June at the beginning of the spawning period. From this high point the weight of the ovary falls off rapidly as the eggs are shed to reach its lowest value in July at the end of spawning, then gradually increasing again to its maximum weight at the beginning of the next spawning period.

From this examination of the gonads of *Fundulus* collected at various times throughout the year it can be seen that the weight and the gross and microscopic appearance of both testis and ovary vary at different periods of the year. The differences are great enough to afford a definite index to the activity of the gonads. By comparing the weight and microscopic appearance of the gonads of experimental animals with this normal series the degree of sexual activity of such experimental animals and their position with reference to the normal sex cycle may be determined.

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THE CHEMICAL NATURE OF GROWTH FACTORS REQUIRED BY MOSQUITO LARVÆ

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Previous work (1–4) has shown that the larvæ of the yellow fever mosquito (*Ædes ægypti*) require certain accessory growth factors which they normally obtain from living microörganisms. A proper quantity of living yeast suspension in distilled water supports good growth of the larvæ, but the same quantity of heat-killed yeast suspension under sterile conditions supports only a very slight growth. If, however, it is supplemented with an adequate amount of autoclaved liver extract, growth proceeds at a maximum rate. Liver extract without yeast gives no growth. Very large amounts of heat-killed yeast suspension (30 to 40 times as much as is required in the presence of liver extract) support fair growth even in the absence of liver extract. Thus at least two substances or groups of substances are required by the larvæ. Only one of these (designated as growth factor A) is present in liver extract. Both are present in heat-killed yeast, but much more yeast is needed to furnish adequate amounts of the one present in liver extract than of the other (4).

The sources and some of the properties of both growth factors have been described in previous publications (2, 4). This paper will show that riboflavin is one of the essential substances supplied by growth factor A and that thiamin (vitamin B_1) is an essential substance supplied by both of the crude growth factor preparations.

METHODS

The Larval Growth Test

All the tests were conducted in 18 × 160 mm. test tubes holding 6 ml. of the medium to be tested. The various constituents of the medium were prepared as separate stock solutions adjusted to a pH of 5.8–6.0 and autoclaved ½ hour at 120° C. The required amounts

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[1] Y. S. was aided by grants from the Milton Fund of Harvard University and the Proctor Fund of Harvard Medical School.

of each solution were then pipetted into each experimental tube and made up with sterile distilled water to a total volume of 6 ml. When the growth factor A activity was being measured, each tube received 0.3 ml. of a washed, killed yeast suspension in distilled water (4).

In some experiments concerned with the factor A activity, and in all the experiments concerned with the other factor and with thiamin, the killed yeast was replaced by yeast extract (yeast vitamin Harris) and casein. Since the yeast extract used contains factor A (4) it was necessary first to treat it for ½ hour with 10 grams of fuller's earth to 100 ml. of 5 per cent solution of yeast vitamin Harris. The fuller's earth was then removed by centrifugation. This procedure removes much of the factor A (4). Amounts of treated yeast extract which supply enough of the second factor when A is supplied in some other form, do not contain enough factor A to support good growth in the absence of added A. In experiments in which whole yeast was replaced by yeast extract and casein, it was also necessary to add Ca^{++} (3), and this was provided as $CaCl_2$ at a concentration of 0.01 M.

Each experimental tube was inoculated with 3 newly-hatched bacteria-free *Ædes ægypti* larvæ secured by the methods previously described (4). Duplicate tubes were prepared for each medium in each experiment. The tubes were incubated at 28 ± 1° C. and were observed daily for the first 10 days and every other day thereafter, the number of larvæ in each stage of development being noted. From the data thus obtained, the rate of development could be expressed as a number, $N \times 1/T$, in which N is the percentage of larvæ reaching the 4th (last) larval instar within 10 days, and T is the average time in days required by these larvæ to reach the 4th instar. The adequacy of this growth index in the measurement of growth factor A has already been shown (4). Under optimum conditions, at 28° C., $N \times 1/T = 100 \times \frac{1}{4} = 25$.

It cannot be too strongly emphasized that significant results with the growth factors here concerned can be obtained only if the larvæ are reared in the complete absence of living microörganisms. To this end, the ordinary bacteriological methods to insure sterility were used and the sterility of each tube was tested by inoculation of a loopful of material to dextrose digest agar. With doubtful or particularly important tubes, broth and cooked meat under vaseline were also inoculated. Contaminated tubes, which occurred but rarely, were discarded.

Preparation of the Flavin-Purine Complex

The 60 per cent alcohol precipitate obtained in the preparation of Cohn's Fraction G (5) was extracted 3 times with 10 volumes of hot

75 per cent acetone. The filtrate was cooled and concentrated. When this was kept overnight in the cold room a heavy precipitate separated out.

This granular yellow precipitate, 20 mg. of which are derived from 100 grams of liver, has a nitrogen content of 28 to 30 per cent. Dr. Max Tishler found that it contains 80 to 85 per cent xanthine by the perchlorate precipitation of Biltz. Mr. Edward Meilman, working with one of us, found it to contain adenosine and sometimes inosine, glutathione, and traces of nicotinic acid amide. The precipitate regularly contains 1.2 per cent flavin phosphoric acid. Its further composition is being investigated.

Preparation of the "Alcohol-Ether Filtrate" and the "Calcium Filtrate"

The charcoal elute described by Subbarow, Jacobson and Fiske (6) was precipitated with 10 volumes of 95 per cent alcohol and 10 volumes of ether. The mixture was left in the cold room for 48 hours and the precipitate filtered off. The filtrate, after removal of the alcohol and ether, was the alcohol-ether filtrate fraction. To prepare the calcium-filtrate fraction, the filtrate was concentrated to a small volume and the concentrate neutralized with $Ca(OH)_2$ and precipitated with 5 volumes of alcohol. The calcium precipitate mostly contained glutathione and adenylic acid. The filtrate was concentrated to remove alcohol. So far, from the filtrate small amounts of ergothionine, tryptophane, tyrosine, nicotinic acid amide, guanosine and ethanol amine have been isolated.

RESULTS

The Resolution of Growth Factor A into Two Fractions

Growth factor A was at first considered to be only one substance, but preliminary attempts at its purification soon indicated that the factor A activity was due to at least two substances. Treatment of a partly purified liver extract in 80 per cent alcohol with barium hydroxide gave a precipitate and a filtrate, each of which, after regeneration, had a very low activity. But combination of both fractions almost restored the original activity.

In a previous paper (2) it was shown that treatment of liver extract by charcoal removes the factor A activity. At that time no successful elution of the active material from the charcoal had been accomplished. Meanwhile, Subbarow, Jacobson and Fiske (6) had obtained a partially purified anti-pernicious anemia material prepared by adsorption on charcoal and subsequent elution with alcohol. This elute gave fairly good growth of the *Ædes ægypti* larvæ only if used at relatively high concentrations, on the basis of the weight of liver from which it was derived. It was then found that if this elute were supplemented with the flavin-purine complex prepared from liver, high values for $N \times 1/T$ could be obtained with much lower concentrations of the elute, although the flavin-purine complex alone had no factor A activity whatever (Table I). In the presence of the high concentrations of elute needed to obtain $N \times 1/T$ values of about 20 in the absence of flavin-purine complex, metamorphosis to the adult stage required a longer time and was accomplished by far fewer insects than in the presence of both elute and flavin-purine complex. A concentration of each of the two components, such that 100 ml. of medium contained that amount of each derived from 50 grams of liver, gave $N \times 1/T$ values of 20 or more, while half this concentration gave values of about 10. These results show that the amount of elute plus flavin-purine complex derived from 50 grams of liver has a factor A activity equivalent to that amount of Lilly liver extract No. 343 derived from about 12.5 grams of liver (a 0.5 per cent solution).

TABLE I

The effect of the flavin complex

Concentration as grams of liver from which was derived amount of fraction present in 100 ml. of culture medium		
Charcoal elute	Flavin complex	$N \times 1/T$
600	0	18.2
400	0	20.0
200	0	13.3
100	0	2.1
200	200	23.7
100	100	25.0
50	50	20.0
27	27	10.1
13.5	13.5	1.7
6.7	6.7	0
0	50	0

The growth factor present in the charcoal elute has been subjected to further purification, the activity going into the alcohol-ether filtrate and the calcium-filtrate fractions prepared as already described. In the presence of suitable concentrations of these more highly purified fractions, the same amounts of flavin-purine complex gave the same growth as in the presence of the charcoal elute itself. The nature of the substances responsible for the activity of the calcium-filtrate fraction is now being investigated.

Comparison of the Effects of Riboflavin and the Flavin-Purine Complex

Repeated experiments have shown that, in the presence of the alcohol-ether filtrate or the calcium filtrate, pure crystalline riboflavin will support excellent growth of the larvæ (Table II). Very much lower concentrations of riboflavin are required than of the flavin-purine complex. In the presence of alcohol-ether filtrate, as little as 0.025 mg. of riboflavin per 100 ml. gave growth almost as good as did 1 mg. of riboflavin, but somewhat less than that obtained with 10 mg. of flavin-purine complex; 0.008 mg. of riboflavin per 100 ml. gave growth as good as 5.4 mg. of flavin-purine complex. In the presence of the calcium filtrate, 0.04 mg. of riboflavin per 100 ml. gave a value for $N \times 1/T$ as high as that obtained with 6.6 mg. of flavin-purine

TABLE II

Comparison of the effects of flavin complex and riboflavin. Each of the tubes contained for lines 1–12, alcohol-ether filtrate, for lines 13–24, calcium filtrate, each at a concentration such that 100 ml. of solution contained the material derived from 53 grams of liver.

Concentration mg. per 100 ml.		$N \times 1/T$	Percentage of larvæ transforming to adults	Percentage of larvæ transforming to normal adults
Flavin complex	Riboflavin			
0	0	0	0	0
20.0	0	23.1	100	100
10.0	0	23.1	67	67
5.4	0	6.5	33	0
0	1.0	16.6	50	0
0	0.5	15.8	33	0
0	0.33	10.7	0	0
0	0.17	10.7	17	0
0	0.08	13.4	33	0
0	0.04	14.1	33	0
0	0.025	14.1	33	17
0	0.008	6.0	33	17
0	0	0	0	0
13.4	0	24.0	100	100
10.0	0	18.9	67	67
6.6	0	23.1	100	100
3.3	0	14.8	50	50
0	0.25	19.8	67	0
0	0.17	21.4	83	0
0	0.125	18.0	67	0
0	0.080	19.8	67	50
0	0.040	22.2	67	33
0	0.017	14.8	67	33
0	0.008	6.2	17	17

complex, while 0.017 mg. of riboflavin was about equivalent to 3.3 mg. of flavin complex. Since the flavin complex contains 1.2 per cent flavin-phosphate, it is readily apparent that the flavin as contained in the complex has about the same activity, so far as larval growth is concerned, as free riboflavin. But it was soon noted that many of the larvæ growing in media containing riboflavin in place of flavin complex had difficulty in metamorphosing into the adult stage. This fact is well shown in Table II. In the presence of alcohol-ether filtrate, those concentrations of flavin complex which gave the highest $N \times 1/T$ values permitted 67 to 100 per cent of the larvæ to emerge as adults and all the adults which emerged were normal and vigorous. Adults were considered normal if they were able to emerge completely from the pupal sheath and to fly up from the surface of the liquid. Abnormal adults were those which either failed to emerge completely from the pupal sheath, or, having done so, were too weak to get free from the surface of the liquid. In Table II it may be seen that in media containing alcohol-ether filtrate and concentrations of riboflavin giving high $N \times 1/T$ values, very few adults emerged and most of these were abnormal. Larvæ which failed to transform into adults usually died, either in the late larval stage or in the pupal stage. In the presence of calcium filtrate, the same differences are apparent between flavin complex and riboflavin. Here again, riboflavin in the most favorable case permitted only 50 per cent of the larvæ to transform into normal adults. With flavin complex, concentrations which gave the fastest larval growth also gave the highest percentage of normal adults, but with riboflavin the highest percentage of normal adults was in general obtained with concentrations which gave submaximal larval growth rates.

Riboflavin is thus an essential substance for the growth of mosquito larvæ. Flavin-purine complex, however, contains some other substance which is possibly of little or no importance for larval growth but is very important for metamorphosis. The nature of this material is unknown. Perhaps the difference between the effects of the flavin complex and the riboflavin may be accounted for by the fact that the flavin in the complex exists as flavin phosphate.

The Need for Thiamin

It has already been shown (2) that if larvæ of *Ædes ægypti* are grown with casein, $CaCl_2$, liver extract, and yeast extract previously treated with fuller's earth, both the factors present in liver extract and those present in the yeast extract withstand 5 hours of autoclaving at 120° C., if each extract is tested in the presence of the other extract autoclaved for only ½ hour. But if both the extracts are autoclaved for 5 hours, the amount of growth obtained is greatly reduced (Table III). When such autoclaved liver and yeast extracts are supplemented with crystalline thiamin in low concentration, growth is restored almost to that obtained with extracts autoclaved for only ½ hour (Table III). This is true whether the casein be free from water-soluble vitamins only, or from both fat- and water-soluble vitamins, although with the latter type of casein, growth is never as good as with the former, possibly because of a deficiency in a fat-soluble growth factor. Table III also shows the interesting fact that a thiamin solution autoclaved for 5 hours is fully as effective as one sterilized by Berkefeld filtration. This can only mean that certain decomposition products of thiamin are as effective as the intact compound. These decomposition products apparently are not formed when liver extract or yeast extract is autoclaved for 5 hours. Presumably, under these conditions, other less easily utilized products are formed. This accords with the fact that growth does go on even to the adult stage in the medium autoclaved for 5 hours and not supplemented with thiamin. The slower rate and greater difficulty with which it proceeds are perhaps the result of a slow synthesis of an essential substance which is readily synthesized from thiamin itself or certain of its decomposition products. The utilization of certain decomposition products of thiamin has been shown for *Phycomyces* by Schopfer and Jung (7), for excised tomato roots by Robbins and Kavanagh (8) and for *Staphylococcus aureus* by Knight (9).

TABLE III

The effect of thiamin. Each tube contained 0.01 M $CaCl_2$.

Type of casein	Treatment of liver extract (0.5% Lilly 343) and yeast vitamin Harris 0.33% after fuller's earth adsorption	Concentration of added thiamin mg./100 ml.	$N \times 1/T$	
			Exp. A	Exp. B
Harris. Free from water-soluble vitamins	Autoclaved ½ hour	0	23.1	25.0
“ “ “ “ “	Autoclaved 5 hours	0	6.2	7.5
“ “ “ “ “	“ “ “	0.80	20.7	16.7
“ “ “ “ “	“ “ “	0.33	17.6	15.4
“ “ “ “ “	“ “ “	0.17	15.4	16.7
“ “ “ “ “	“ “ “	0.08	—	18.2
“ “ “ “ “	“ “ “	0.33 —autoclaved 5 hours	18.8	20.0
Harris. Free from water- and fat-soluble vitamins	Autoclaved ½ hour	0	18.8	14.3
“ “ “ “ “ “ “	Autoclaved 5 hours	0	0	0
“ “ “ “ “ “ “	“ “ “	0.80	12.8	9.6

DISCUSSION

Early in the study of the nutritional requirements of insects, it became apparent that yeast and other microörganisms were the natural source of certain necessary foodstuffs (10–14). Several workers, as for example Bacot and Harden (15), working with *Drosophila*, and Richardson (16) with *Ephestia kuehniella*, soon found that yeast extract would supply substances essential for the growth of insect larvæ. Since yeast extract was at that time synonymous with vitamin B, these authors concluded that insects require vitamin B. Similar evidence for the vitamin B requirement of insects has been recently supplied by McCay (17) for the roach *Blatella germanica*, by Crowell and McCay (18) for larvæ of the clothes moth *Tineola bisseliella* and by Frost, Herms and Hoskins (19) for larvæ of the mosquito *Theobaldia incidens*. Hobson (20), and Street and Palmer (21) were among the first to show that the yeast extract supplied several different essential substances, and it was Hobson who found that pure vitamin B_1 is one of these substances. Van't Hoog (22) has gone still further and shown that both crystalline riboflavin and thiamin are substances essential for the growth of larvæ of *Drosophila melanogaster*. Growth does not occur, however, unless still a third substance or group of substances, also present in yeast extract, is supplied. Thus the water-soluble accessory growth factors required by *Drosophila melanogaster* and *Ædes ægypti* are identical so far as they are known at present. Both insects require for larval development riboflavin, thiamin, and a third factor present in yeast extract, liver extract, rice-polishings concentrate, etc. It is interesting that these requirements form a perfect parallel to the vitamin B complex requirements of vertebrates (23–25).

The nature of the third factor required by vertebrates and present in liver extract is now under investigation in several laboratories. The indications are that nicotinic acid amide or cozymase is one of the essential substances present in the third factor, but not the only one (26–29). Lepkovsky (30) has recently isolated one of these substances in crystalline form, but he gives no information as to its nature or method of preparation. So far as *Ædes ægypti* larvæ are concerned, nicotinic acid or its amide cannot replace the "calcium-filtrate" fraction of liver extract. But evidence is now accumulating that nicotinic acid amide plus certain other substances can at least partially replace the impure liver fraction.

It is worth noting that while rice-polishings concentrates have been generally considered to be free from riboflavin (as in the work of Cook, Clarke and Light (28)), they must actually contain appreciable amounts, since a 0.4 per cent solution of Ryzamin B has been found (4) to give growth about as good as a 0.3 per cent solution of Lilly liver extract No. 343. On the basis of this result, a 0.4 per cent solution of Ryzamin B should contain around 0.02 mg. of riboflavin per 100 ml. It is not surprising that the clay adsorbate (the International Standard vitamin B_1) prepared from rice-polishings concentrate also can replace liver extract (4), since the flavin and other necessary factors would be adsorbed as readily as the thiamin.

The authors thank Dr. Guy W. Clark of Lederle Laboratories for supplying liver extract for the chemical work. W. T. is grateful to Dr. R. W. Glaser for his helpful interest in the work.

SUMMARY

Riboflavin, and thiamin or certain of its degradation products, are necessary for the normal development of larvæ of the yellow fever mosquito *Ædes ægypti*. In the presence of all the other necessary growth factors, riboflavin permits normal larval growth at concentrations as low as 0.0004 mg. per ml. and has a definite effect at a concentration of 0.00008 mg. per ml., while thiamin permits normal growth at concentrations at least as low as 0.0008 mg. per ml. Although larval growth is normal in the presence of suitable amounts of heat-killed yeast, riboflavin, and the other liver extract factors supplied in partially purified form, metamorphosis to the adult stage is successfully accomplished by a relatively small percentage of the insects. If, however, the flavin is supplied as a flavin-purine complex containing 1.2 per cent flavin-phosphate, almost all the larvæ transform into vigorous adults. The nature of the additional factor responsible for this difference is not known.

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COPULATION IN THE ACŒLOUS TURBELLARIAN POLYCHŒRUS CARMELENSIS[1]

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Attention has been directed recently to the primitive condition of the reproductive system of the Turbellaria Acœla, as compared with most of the Platyhelminthes, and to the occurrence of copulation in the group (Hyman, 1937). The Acœla are hermaphroditic. Most members of the group possess a copulatory mechanism including an extensible penis and a seminal receptacle, provided with one or more small tubes called "Mundstücke," the function of which is assumed to be the transport of sperm from the receptacle or bursa toward the eggs. In view of the presence of a copulatory mechanism, von Graff (1905) assumed that true copulation is of widespread occurrence in the group.

Gardiner (1898) had earlier concluded that in *Polychœrus caudatus* sperm transfer is accomplished by a process of hypodermic impregnation, in which the "mouth pieces" function as hypodermic organs. Bresslau (1933) and Hyman (1937) admit that hypodermic impregnation may be of common occurrence in the Acœla. We have found no specific account of this process other than that of Gardiner (1898). Only two descriptions of true copulation in the Acœla have come to our attention, that of Peebles (1915) for *Monochœrus lineatus* and that of Hyman (1937) for *Amphiscolops langerhansi.* Only in the latter case was the observation of living animals supplemented by the study of sectioned pairs. The study of both living and sectioned specimens of *Polychœrus carmelensis*, a form very closely similar to *P. caudatus*, has yielded no evidence of the occurrence of hypodermic impregnation. The evidence demonstrates conclusively that impregnation is regularly accomplished by means of true copulation, the details of the process differing considerably from that described by Hyman for *Amphiscolops langerhansi*.

During the summer months of 1936 and 1937 *Polychœrus carmelensis* were collected in great abundance from tide pools at Pescadero Point on Carmel Bay, California, and in smaller numbers from tide pools elsewhere in the vicinity. Examination of living and sectioned specimens soon revealed certain structural differences from the Atlantic

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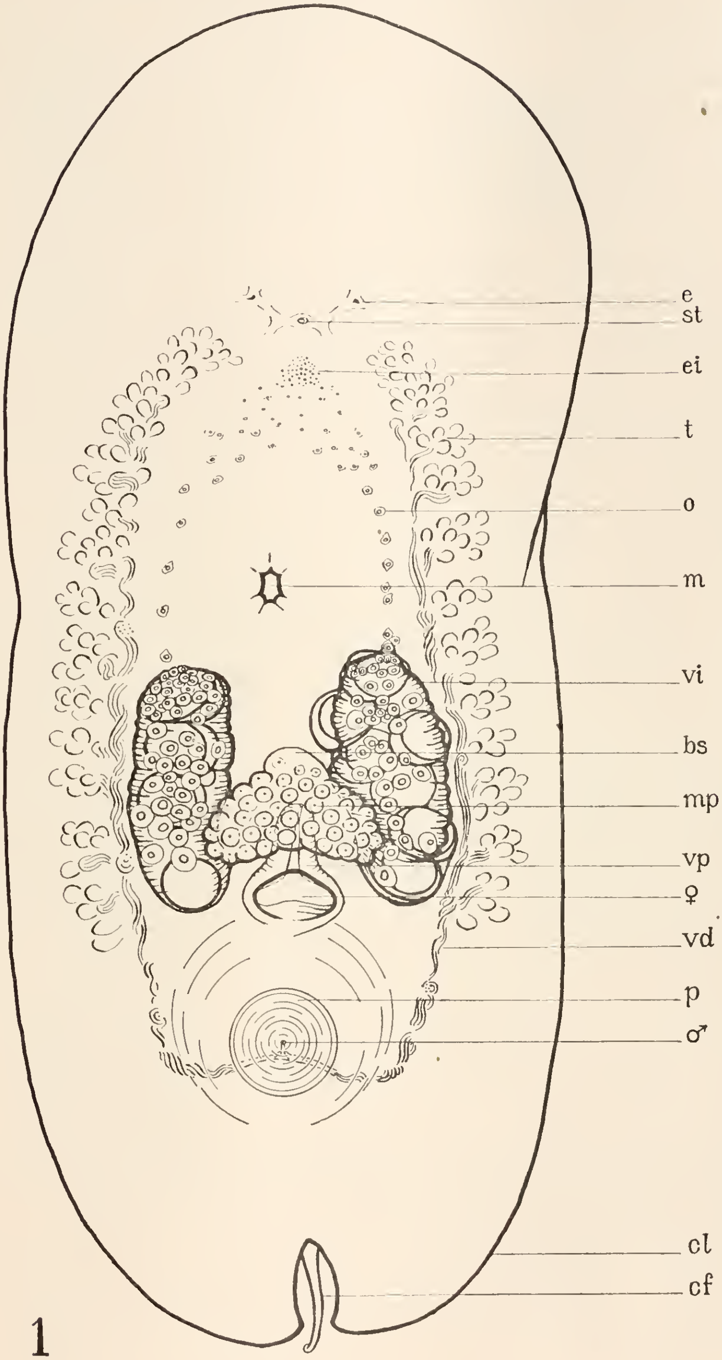
[1] The authors are indebted to the Society of Sigma Xi for aid in the publication of this work.

Coast *Polychœrus caudatus*, as described in the papers of Mark (1892), Verrill (1893), Gardiner (1895, 1898), and Löhner (1910). On the basis of these differences the Pacific Coast form was designated as a distinct species (1938).

A brief description of the reproductive system of *P. carmelensis* will be presented in order to facilitate an understanding of the copulatory process. Previously, the reproductive system has been discussed only with respect to certain points in which it differs from that of *P. caudatus* (1938).

The reproductive structures of *P. carmelensis*, in ventral view, are illustrated diagrammatically in text fig. 1. The female system includes a pair of gonads which appear to originate anteriorly in a median "Eilager" (*ei*), a short distance posterior to the statocyst (*st*). Extending posteriorly from the Eilager are two lateral ventral rows of oöcytes (*o*) which, a short distance below the level of the mouth, become embedded in the vitellaria (*vi*), the latter terminating at the level of the female aperture (♀). In the vitellaria the young oöcytes are located ventrally, and move dorsally as they mature. The female gonads are typical of the Acœla in their lack of specialized investing walls and of ducts to either the exterior or to the bursa seminalis. The external female aperture is roughly elliptical, about one-sixth the width of the body, enormously distensible. From this aperture there extends dorsally, to a distance of about two-thirds the thickness of the body in this region, a large dorso-ventrally flattened chamber, the vagina. The anterior wall of the vagina bears, at a position somewhat dorsal to its mid-point, a thick-walled pocket (*vp*) which is directed anteriorly, slanting slightly dorsally, and provided with a small lumen. The anterior end of the pocket is embedded in the dorsal part of the bursa seminalis (*bs*), its lumen opening directly into the bursa much in the manner in which Mark described the vagina of *P. caudatus* as communicating at its dorsal end with the naked bursa tissue. We find in the literature no mention of a structure in *P. caudatus* corresponding to the vaginal pocket of *P. carmelensis*. The muscular walls of the vagina and pocket are lined with a nucleated epithelium lacking cilia. These structures are shown, in sagittal section, in plate figures 1 and 2.

TEXT FIG. 1. Ventral view, showing arrangement of male and female reproductive systems. Composite drawing, with projector, from two whole mounts stained with Delafield's hæmatoxylin, the testis follicles largely diagrammatic. Magnification 35 ×. *bs*, bursa seminalis; *cf*, caudal filament; *cl*, caudal lobe; *e*, eye; *ei*, "Eilager"; ♀, external female aperture; ♂, external male aperture; *m*, mouth; *mp*, mouth piece of bursa; *o*, oöcyte on way from Eilager to vitellarium; *p*, penis bulb; *st*, statocyst; *t*, testis follicles; *vi*, vitellarium containing young oöcytes and mature eggs; *vd*, vas deferens; *vp*, vaginal pocket.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The bursa seminalis appears to resemble that of *P. caudatus* in all essential features. It is somewhat kidney-shaped, and is located medially between the two vitellaria, anterior to the female aperture and the vagina. It occupies the space between the vaginal pocket and the ventral body wall but extends anteriorly well beyond the pocket, the anterior dorsal portion surrounding the anterior tip of the pocket. The internal structure of the bursa is shown, in slightly oblique transverse section, in plate fig. 3. The entire ventral part of the bursa is composed of many small compartments, each containing a small tubular "mouth piece" (*mp*), the long axis of which approximately parallels the dorso-ventral axis of the body. The ventral small ends of these tubes open into the vacuolated marginal parenchyma (*va*), a thin layer of which separates the bursa from the ventral integument. At the basal end of each mouth piece is usually attached a mass of sperm (*s''*) occupying a small vacuole. The ventral compartments of the bursa merge dorsally into a chamber (*bs'*) which frequently contains scattered sperm masses sometimes connected with the ventral masses.

Regarding the histology of the bursa a few facts may be mentioned. The syncytial tissue of which the bursa is composed sometimes appears almost continuous (plate fig. 3) except for the presence of sperm masses, muscle fibers and a few small vacuoles. The highly vacuolated appearance, especially of the dorsal part of the bursa, which seems to characterize many specimens (plate fig. 2), probably results largely from the disruptive influences of mating and egg-deposition, although the method of fixation is to some extent responsible, slowly penetrating fixatives permitting time for violent muscular contraction and consequent distortion of tissue. The bursa lacks a differentiated limiting wall. Ventrally, it impinges upon marginal parenchyma (in the terminology of Löhner); dorsally, it is bounded by the anterior pocket posteriorly and central parenchyma anteriorly (plate figs. 1 and 2); its anterior curved surface approximates the intestinal parenchyma (*ip*) from which it is separated by muscle fibers; laterally, it is enclosed by the vitellaria. The bursa communicates with the exterior by way

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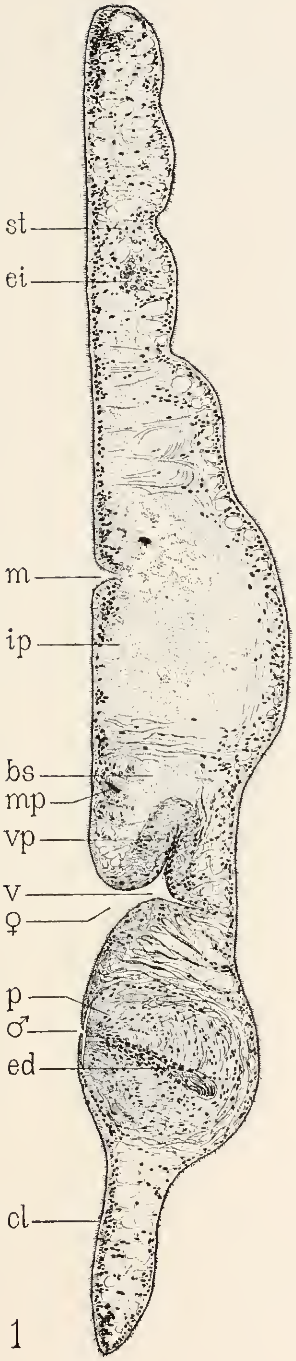
PLATE I

Median sagittal sections of a non-copulant individual (Fig. 1) and a copulant individual (Fig. 2), for comparison of the genital regions. Drawn with aid of projector. *ed*, ejaculatory duct; *f*, food organism; *ip*, intestinal parenchyma; *s*, newly-acquired sperm; *v*, vagina; *v'*, opening of lumen of vaginal pocket into vagina; other abbreviations as used in text fig. 1.

FIG. 1. Fixed in Heath's solution, stained with Heidenhain's hæmatoxylin. Magnification 51 ×.

FIG. 2. Fixed in Champy's fluid, stained with Heidenhain's hæmatoxylin. Magnification 47 ×.

PLATE I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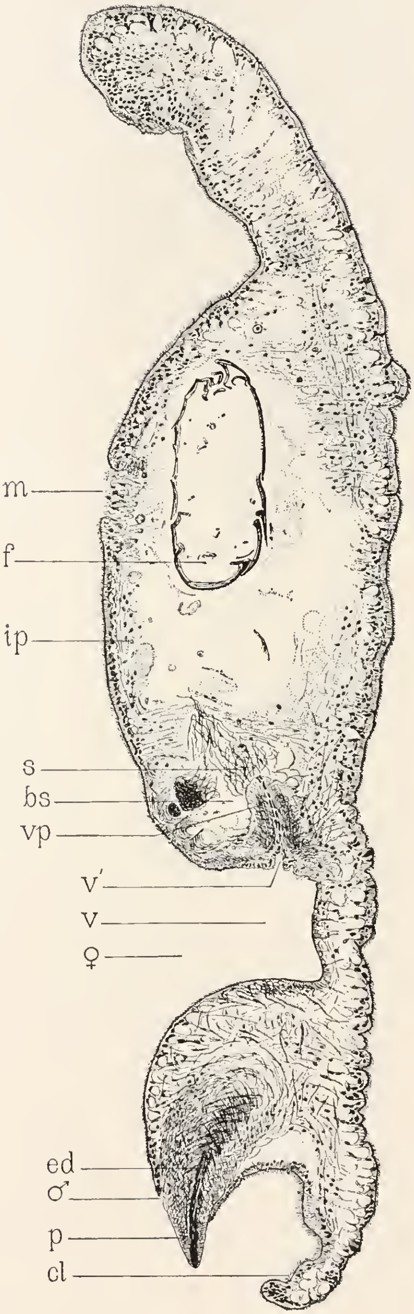
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of the small lumen of the vaginal pocket, the vagina and the female aperture.

The male reproductive system (text fig. 1) includes a pair of gonads of follicular arrangement (*t*), extending from approximately the level of the statocyst to a point somewhat posterior to the female aperture. They are lateral and dorsal to the female gonads. The vasa deferentia (*vd*) are a pair of channels in the parenchyma through which sperm pass from the follicles of each side to the ejaculatory duct (plate figs. 1 and 2, *ed*) of the penis (*p*). The testes and vasa deferentia lack specialized investing walls; the ejaculatory duct is lined with a nucleated epithelium. The penis is located posterior to the vagina. It is compact, muscular; in the retracted state, an ovoid bulb, which occupies almost the entire thickness, and nearly one-fourth of the width, of the body in this region (plate fig. 1, *p*). Its dorsal end is inclined somewhat posteriorly. In the retracted state the ventral tip of the penis extends free into a small circular chamber which is covered by ventral integument bearing a very small, but greatly distensible, perforation, the male aperture (♂). During activity the penis is protruded through the male pore, and assumes an elongated tapering conical form, arched so that the distal end is directed posteriorly (plate fig. 2, *p*).

The copulatory mechanism of *P. carmelensis* consists, in brief, of the male organ and aperture, the female aperture, the vagina with anterior pocket, and the bursa or seminal receptacle.

Material and Procedure

Collections of *Polychœrus carmelensis* were brought into the laboratory on the *Ulva* (and a few other sea-weeds) upon which they are found in the tide pools. Large numbers of individuals of all ages were removed immediately from the *Ulva* and preserved for later study. Usually a few hundred worms were distributed among several finger-bowls. Most of the remainder of the *Ulva*, bearing thousands of worms and abundant food organisms (chiefly small crustacea), was placed into two running salt-water aquaria. Here the worms remained alive and active for long periods of time.

During the summer of 1936 our attention was focused mostly on

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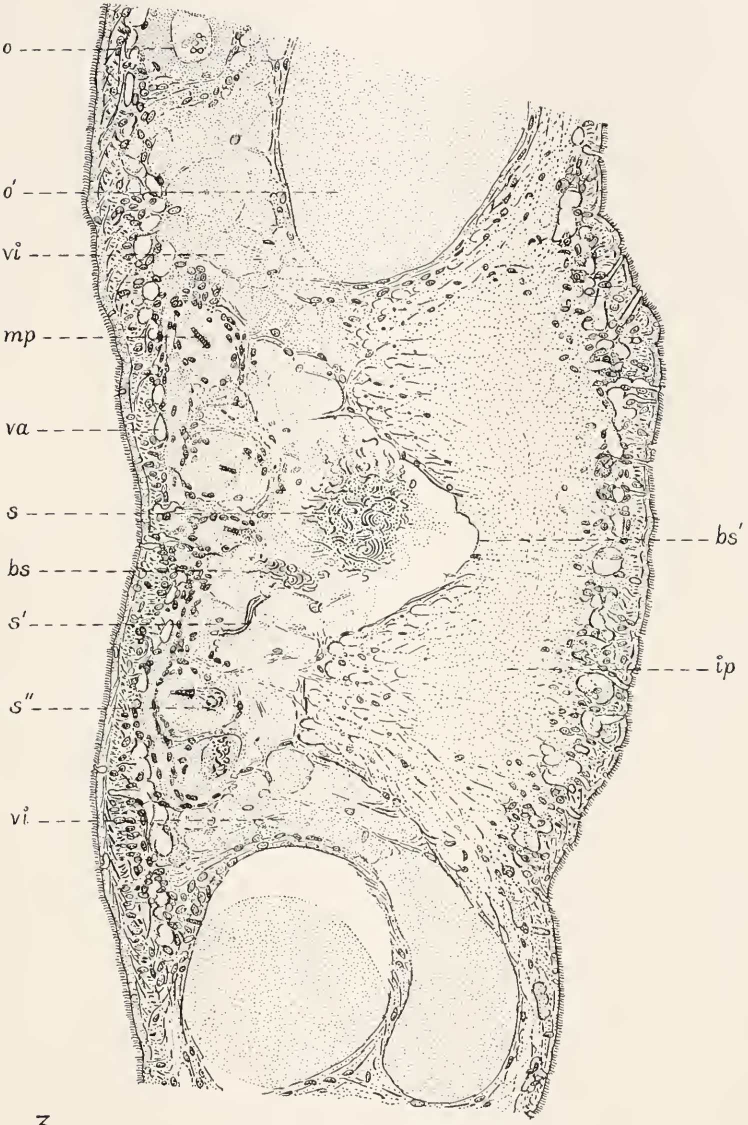
Plate II, Figure 3

Detailed portion of section of text fig. 3 (*a–b*), to show newly-acquired sperm mass (*s*) in the dorsal part of the bursa anterior to the vaginal pocket. Drawn with aid of projector. Magnification 180 ×. *bs*′, surface of dorsal chamber of bursa; *o*, young oöcyte, and *o*′, nearly mature egg, in vitellarium; *s*′, sperm, apparently migrating from mass (*s*) toward ventral bursa mouth piece; *s*″, sperm ball attached to mouth piece; *va*, vacuole of ventral marginal parenchyma; other abbreviations as previously used.

Plate II, Figure 3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a study of the anatomy and development of *P. carmelensis*. All observations of mating activities were made in 1937, in the period between June 24 and August 24. Observations were rendered somewhat difficult by the extremely brief duration of mating; the entire process rarely exceeded a minute and a half. In all, 35 matings were witnessed; most of these were observed in detail, usually with the aid of a hand lens. Occasional pairs were removed to glass slides for immediate examination, and fifteen pairs were fixed for later sectioning and study. The pairs were transferred by means of a pipette from aquarium or fingerbowl to the fixative. Unfortunately, the mates separated in every case, either during this transfer or at some time before the embedding process was reached. This separation removed the possibility of subsequent identification of members of a single pair, since two or more pairs were usually stored in a single vial. Only two pairs were stored separately. However, the data furnish ample evidence with regard to the essential features of the mating process in *Polychœrus carmelensis*.

OBSERVATIONS

Copulation was most frequently observed on the glass sides of the aquaria where the worms were present in great numbers. About one-third of the observed matings occurred in the fingerbowls. Mating was never seen in the tide pools, but is assumed to have preceded the deposition of eggs which were found in abundance on small stones and in the folds of *Ulva*. In *Polychœrus carmelensis* the eggs are fertilized before deposition, and there is no evidence of the occurrence, in this species, of self-fertilization or of any method of sperm transfer other than true copulation.

The 35 matings witnessed in the laboratory are listed in Table I, according to the respective dates and hours of occurrence. The table shows that matings are infrequent during the late morning, afternoon, and evening, but are relatively numerous immediately after dawn, from 5 to 7 o'clock. Periods of observation of the aquaria and fingerbowls included all hours of the day and night except from 1 to 4 A.M. However, in the table, hours of observation (+) are indicated, where records are sufficiently complete, only for days upon which one or more matings was witnessed. While observations during the period from 4:45 to 9:30 A.M. were made only on the three dates listed, the observations during the usual working hours of the day, as well as at night, were far more extensive. Therefore, the difference in frequency of mating in the early morning as compared with the later hours is probably greater than the total numbers indicate. Indeed, after having repeatedly watched in vain for signs of mating, the occurrence of nine or ten matings within 2 to 2½ hours after dawn seemed a veritable epidemic. It is interesting to note that, whereas early morning is the preferred mating time, egg-deposition occurs usually after sundown.

There was considerable variation in the degree of activity of the worms during the month of observation, probably in some measure correlated with the length of time during which they had been kept in the laboratory. However, our data are of insufficient extent to provide evidence of this correlation.

The process of mating took place in the following manner (text fig. 2). Considerable restlessness was evidenced by certain mature-looking worms, which usually contained visible accumulations of sperm in the vasa deferentia and at the base of the ejaculatory duct.

Such individuals moved about actively, frequently elevating the head end, and pausing to exchange head contacts with other worms. In case such overtures between two worms were prolonged, the individuals faced each other with the anterior halves of their bodies touching ventrally and swaying slowly from side to side in opposite directions (2*a*). This process was sometimes terminated by one worm moving away. If continued, one animal presently bent its anterior half down under its partner, twisting at the same time so as to bring its ventral surface into contact with the ventral surface of its partner, and retaining attachment to the substratum along the posterior margin of one side. The partner at the same time arched dorsally, elevated the side adjoining the other individual, remaining attached only along the margin of one side (2*b*). The two worms then glided along each other in opposite directions, ventro-lateral margins in contact, until each

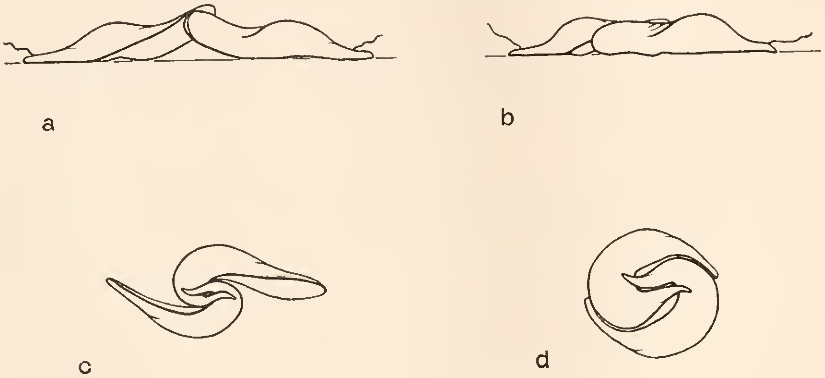
TABLE I

The numbers of matings observed in the laboratory are listed according to the dates and hours of occurrence. The hours of observation (+) on the respective dates are indicated, where the records are sufficiently complete.

	4:45–5 A.M.	5–6	6–7	7–8	8–9	9–10	10–11	11–12	12–1 P.M.	1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	Total observed matings
June 24........											1							1
July 28........						3												3
29........								1										1
31........	+	5	4	+	+													9
Aug. 2........						+	+	+				2					1	3
9........	+	4	3	3		3					+	+	1			1	+	15
13........							+	+	+	1	+							1
16........						+	+	+	+	+	1	+						1
24........		1	+	+						+	+	+	+					1
Total observed matings		10	7	3		6		1		1	2	2	1			1	1	35

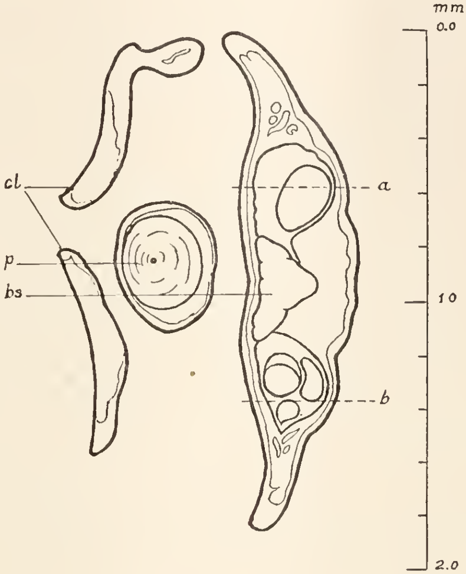
animal had passed more than half its length beyond its partner. At this point, when the copulatory organs were presumably in juxtaposition, one animal, with a sudden snap, released its attachment completely, both curled ventrally, the pair forming a ball attached only by the lateral marginal papillæ of one animal (2*c* and 2*d*). After a connection of only 40 to 50 seconds, the attached animal uncurled, and began to move off, necessitating the reattachment of its partner. The entire mating process, including overtures, occupied only 1 to $1\frac{1}{2}$ minutes.

The mating position obviously conceals the actual relation of the genital regions of the two worms. Mating pairs placed on slides for examination separated immediately. However, they showed certain facts clearly. The genital region invariably evidenced activity by the protrusion of the penis, the repeated contraction and distention of the vagina and the female aperture. In several individuals a large mass of sperm was visible in the dorsal part of the bursa anterior to the vaginal pocket. Only once were sperm observed at the female aperture; there was never a trace of sperm in the vagina, pocket, or about the penis, though a fluid filled with oil-like globules issued from the penis of one individual. The presence of sperm in the bursa, and their complete absence from vagina, pocket or penis, suggested that sperm might be deposited directly into the dorsal chamber of the bursa by the insertion of the penis of one worm into the lumen of the vaginal pocket of its mate. Confirmatory and supplementary evidence was provided by the sectioned material.



TEXT FIG. 2. Series of sketches showing progressive stages in the assumption of the typical copulatory position. *a*, *b*, mating pair viewed in plane parallel to the substratum; *c*, *d*, viewed in plane perpendicular to the substratum.

A sagittal view of a typical individual fixed during copulation (plate fig. 2) shows striking differences from the typical non-copulant (plate fig. 1). The body is usually curved ventrally though seldom retaining in fixation the full flexure of the mating position. Invariably, the penis (*p*) is protruded through the male aperture (♂), and the vagina (*v*) distended. The vaginal pocket (*vp*) is usually curved slightly ventrally, the epithelial lining of the lumen showing signs of activity in the antero-posterior orientation of the nuclei and the pseudopodial projections and occasional sloughing at the opening into the vagina. In the non-copulant the penis is retracted, the vagina and female aperture antero-posteriorly flattened. The pocket infrequently displays evidence of activity, and in some cases the lumen is not visible at the anterior end. A few individuals, such as that shown in text fig. 3, in transverse section, retained more completely the curvature of the typical copulatory position (text fig. 2, *c* and *d*). The body is bent ventrally at the level of the vagina, so that the transverse section through the anterior portion of the bursa (*bs*) passes also through the extended penis (*p*), and through the caudal lobes (*cl*). Thus, the copulatory organs of the fixed mating worms indicate activity and are in such a position as to permit the insertion of the



TEXT FIG. 3. Transverse section (slightly oblique dorso-ventrally) of individual fixed, during copulation, in Heath's solution and stained by the Flemming tri-color method. The ventral curvature typical of the copulatory position is shown by the presence of caudal lobes (*cl*), and penis (*p*), in section which passes through the anterior portion of the bursa (*bs*). Camera lucida drawing, with scale.

penis of each individual into the anteriorly-directed pocket of its mate.

The accomplishment of impregnation during copulation is evidenced by 16 of the sectioned worms. In these animals (plate figs. 2 and 3) a large mass of sperm (*s*) is found in the dorsal part of the bursa usually anterior to the pocket, but frequently connected by strands of sperm with the anterior end of the lumen. The absence of sperm elsewhere in the lumen, and in the vagina, in both fixed and living copulants, seems to justify the conclusion that the sperm are deposited directly into the dorsal chamber of the bursa by the insertion of the penis of the mate into the vaginal pocket, probably as far as the opening of the latter into the bursa. The large aggregates of sperm in the dorsal bursa are sometimes densely coiled (plate fig. 3), sometimes more scattered (plate fig. 2). They are embedded in the bursa tissue; several cases in which they appear to be embedded in the intestinal parenchyma probably represent distortion due to slow fixation. The enormous size of these sperm masses, their position anterior to the pocket, and their frequent connection with the pocket lumen, distinguish them from those found in random specimens. They are without doubt masses deposited during the mating which was terminated immediately before, or interrupted by, fixation. Three additional sectioned worms contained smaller masses of sperm which were judged to be newly-acquired. The remaining eleven worms gave no evidence of impregnation.

It is obvious that some of the fifteen pairs of worms were reciprocally impregnated since more than half of the individuals contained newly-acquired sperm. The two mating pairs which were preserved in separate vials were both cases of reciprocal impregnation. By noting for each vial in which more than one mating pair were preserved, the proportion of individuals containing newly-acquired sperm, it was found that at least six of the total fifteen matings involved reciprocal impregnation. Allowing for the disturbance of the mating worms incident to their transfer to the fixative, it appears probable that reciprocal impregnation is of more usual occurrence than the minimum figures indicate.

Thus, the results of examination of the fixed material are in harmony with, and supplement, the observations of the living mating worms. There was no evidence of the use of bursa mouth pieces for the accomplishment of sperm transfer by the hypodermic method, as postulated by Gardiner for *P. caudatus*.

The subsequent path of the sperm from the dorsal part of the bursa to the eggs will be considered only briefly at present. Undoubtedly, some of the sperm migrate ventrally within the bursa to the mouth pieces, as evidenced by certain of the fixed copulants (plate fig. 3, *s′*). This is in accord with Mark's view of the distribution of sperm in the bursa of *P. caudatus*. We have as yet found no evidence of the passage of sperm through the mouth pieces to the eggs by way of the vacuolated ventral parenchyma, as Löhner postulated for *P. caudatus*. We hope to consider this subject more fully in a paper dealing with the maturation and fertilization of the eggs of *Polychœrus carmelensis*.

Discussion

Attention may be directed to certain similarities and differences in the copulatory process of the three species of Acœla for which it has been described, *Monochœrus lineatus*, *Amphiscolops langerhansi*, and *Polychœrus carmelensis*. The general copulatory position is similar in all cases in that the mates are united by the ventral surfaces of the posterior ends, their anterior ends extending in opposite directions. No further details of the process were given by Peebles (1915) for *Monochœrus lineatus*. In *Amphiscolops langerhansi* (Hyman, 1937), the mates assume the form of a ball for about thirty seconds prior to the assumption of the final mating position, in which they are unrolled and attached by their ventral surfaces. The ball form is closely similar to the final mating position characteristic of *P. carmelensis*. The prolonged union of forty to fifty-five minutes in *Amphiscolops* contrasts strikingly with the extremely brief union of forty to fifty seconds in *P. carmelensis*.

The relative positions of the reproductive organs of the mates differ, in the two species, in correspondence with structural differences. In *Amphiscolops*, the bursa opens into the female genital pore by way of a small invagination (Hyman, p. 322). The penis papilla is free only posteriorly, behind which there is a projecting sperm guide. Copulation occurs by the interlocking of penes. The sperm are directed toward the bursa of each individual by the sperm guide of the mate. Impregnation is mutual in *Amphiscolops langerhansi*.

Summary

The process of mating is described for *Polychœrus carmelensis*. A study of living and fixed mating individuals, separated from their mates, indicates that copulation involves the insertion of the penis of each individual into the vaginal pocket of the mate, so that sperm are deposited directly into the dorsal chamber of the seminal bursa. Impregnation is, in some cases, reciprocal. The entire process lasts only one to one and a half minutes.

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TRANSPLANTATION OF MUTANT AND WILD TYPE BRISTLE-BEARING TISSUES IN DROSOPHILA MELANOGASTER[1]

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Introduction

The data on mosaics (Sturtevant, 1932) show that certain bristle mutants such as scute and yellow, though ordinarily autonomous, may display non-autonomous development if present in small patches of tissue. Development of such characters as forked, singed and spineless, however, is not influenced by surrounding tissues of a different genotype (Morgan, Bridges and Sturtevant, 1919; Patterson, 1931).

Imaginal disks from which arise the bristle-bearing regions of adult flies are, on transplantation to host larvæ, known to reach a maximum degree of development. Their condition indicates that they may serve as a basis for the study of genic interaction in the same manner as have imaginal eye tissues (Ephrussi and Beadle, 1937; Beadle and Ephrussi, 1936).

Since the effects produced by imaginal eye disk transfer have led to the postulation of definite diffusible substances active in the production of eye color, it is reasonable to assume that the presence of similar substances, essential for the development of various bristle types, may be demonstrated. Plunkett (1926) has postulated that there may be something which, spreading from a region of maximum effect, tends (*a*) to remove bristles or (*b*) to form bristles. Additional evidence of the existence of such a substance is offered by Child (1935).

Materials and Method

The technique used in making transplantations in *Drosophila* has been so fully described (Ephrussi and Beadle, 1936) that a detailed repetition is unnecessary. An imaginal disk, dissected out of one larva, the donor, was picked up in a micro-pipette and injected into the body cavity of the etherized host. Generally the operations were carried out with larvæ twenty-four hours prior to puparium formation (so-called "standard" age). When the fly had hatched, it was dis-

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[1] Grateful acknowledgment is made to the American Philosophical Society whose grant-in-aid made the work herein reported possible.

sected in a 20 per cent solution of glycerin in alcohol, this medium being used to keep the bristles flexible and so prevent their breaking. After the various regions of the implant had been identified, it was then unfolded and examined under a compound microscope. If protected by a coverslip, such temporary mounts could be kept for weeks with an occasional addition of small quantities of the glycerin-alcohol mixture.

Another series of experiments was concerned with the injection of body fluid from prepupæ and pupæ into mature larvæ, prepupæ and pupæ. The technique used was similar to that of Beadle, Clancy and Ephrussi (1937) but differed in one respect. Newly-formed prepupæ were caused to adhere to slides by the application of a small amount of culture medium which acted as a glue. In this way it was possible to carry out operations without holding the pupa case. All injections were made posteriorly in order not to injure the macrochætæ-bearing regions.

Description of Stocks Used

The stocks were available from those maintained at Washington Square College, with the exception of the scute-1 stock which was obtained from Dr. G. P. Child of Amherst College. The characters of the various stocks used are briefly summarized below:

(1) + —— wild type, Woodbury and Oregon R strains. Bristle characters in the Woodbury stock somewhat variable, duplications and absences more frequent than in Oregon R.

(2) f (B) —— forked. Macrochætæ forked, gnarled or bent, the posterior scutellars showing the character in most extreme form. Microchætæ unaffected.

(3) sn^3 —— singed. Macrochætæ gnarled, curved and twisted, especially the posterior scutellars. Microchætæ also affected.

(4) ss —— spineless. All macrochætæ reduced in size, especially the scutellar, ocellar and post-vertical bristles.

(5) H^2 ext —— Hairless extreme. An extreme form of Hairless, practically all bristles and hairs missing. Only 50 per cent of the offspring H^2 ext. Stock maintained by constant selection. Trichopores (circular pits bordered by raised annuli) present.

(6) sc —— scute-1. Scutellar bristles nearly always missing, orbitals and post-verticals often gone, presuturals frequently absent. No trichopores present.

(7) y sc —— yellow scute-1. More variable than the above scute-1. One or more scutellar bristles found in about 35 per cent of the flies. Bristle color as well as body color yellow.

Experimental Data

Growth and Differentiation of Implanted Dorsal Mesothoracic Disks

The dorsal mesothoracic disk gives rise to the wing, the dorsal half of the thorax (except the humerus), and half of the scutellum (Chen, 1929). If both host and donor larvæ used are mature, well-developed bristles are found on the implant when it is dissected out of the newly hatched fly. The thoracic and scutellar regions of an implant are a light tan while the wing is gray. If allowed to remain in the host fly for a few days, the implant becomes much darker in color. Younger disks transplanted into hosts of "standard" age produce bristles which are not sufficiently well developed for a critical analysis to be made of the mutant character being studied. However, if such an implant be allowed to remain in the host fly several days after hatching, further development of the bristles will take place.

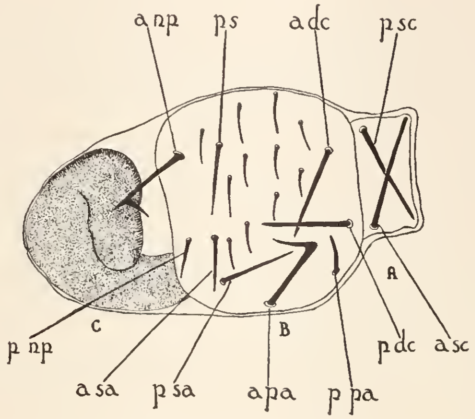

Fig. 1. Diagrammatic representation of a wild type implant showing bristle arrangement. Note the two bent bristles. The wing has been moved to one side and partially unfolded, but the thoracic region has not been touched. *A*, the scutellum; *B*, the thoracic region; *C*, the wing. The macrochætæ have been identified: *a sc*, anterior scutellar; *p dc*, posterior dorso-central; *p pa*, posterior post-alar; *a pa*, anterior post-alar; *p sa*, posterior supra-alar; *a sa*, anterior supra-alar; *p np*, posterior notopleural; *p sc*, posterior scutellar; *a dc*, anterior dorso-central; *ps*, pre-sutural; *a np*, anterior notopleural.

The implant on being dissected out of the host fly is found enclosed in a sac. Since the disk has not been everted during development, it should be carefully opened and spread out for critical observation of the bristles. The eleven macrochætæ developed by each dorsal mesothoracic disk include all the thoracic bristles except the humerals, which develop from the prothoracic disk (Chen, 1929). Size and arrangement of bristles with respect to each other and to the microchætæ make it possible to identify each of them positively in a favorable implant (Fig. 1). If the action of bristle-removing genes, or of genes which cause the growth of extra bristles is being studied, a count of the macrochætæ is sufficient. The scutellar region is ordinarily easy to recognize, unless the scutellar bristles are absent as in scute-1 or Hairless extreme. In these mutants a study of the macrochætæ is essential in the identification of the phenotype of the implant.

Wild Type Disks in Mutant and Wild Type Hosts

Before the results obtained on implantation of bristle-bearing tissues into mutant hosts are reported, it is essential that certain factors which complicate the interpretation of the data be reviewed: It has been previously pointed out (Howland and Glancy, 1937) that wild type implants in wild type hosts exhibit a varying number of bristle types. One of these is simply bent, others strikingly resemble those of the mutants forked and singed, and still others are not identical with the bristle forms characteristic of any known mutant in *Drosophila*.

An analysis of the possible causes of this phenomenon indicated that these effects were the result of pressure exerted on the bristles during their development within the implant. It is evident that the effect of compression on the bristles must constantly be kept in mind, especially if bristle-bearing tissues are allowed to develop in mutant hosts such as forked and singed.

Wild type disks implanted into H^2 ext, ss, y sc, sc, f and sn^3 hosts are held to develop autonomously, since implants in f and sn^3 hosts produce no more forked and singed bristles than are found on the controls. Three or four of the larger bristles may show some distortion and one or two of the smaller ones may also be affected (Howland and Glancy, 1937).

The phenotype of all host flies was normal with the possible exception of a slight modification of the H^2 and y sc. In the former,[2] if the host on hatching proves to be H^2 ext, it is easy to say with certainty that the implant has had no effect, but in H^2 hosts the normal variability in bristle number leaves the matter open to question. Statistical treatment of large numbers would be required to determine beyond any doubt whether a greater number than the normal expectation had been induced to grow.

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[2] It must be remembered that in this stock only 50 per cent of the offspring are H^2 ext, the other 50 per cent being H^2. (See description of stocks.) If used as hosts, the actual condition (H^2 or H^2 ext) can be checked on hatching. When choosing larvæ for donors from this stock there is no way to insure the selection of an H^2 ext mutant. On counting many hundreds of flies from the stock bottles it was found, however, that of the first flies hatching a far greater number were H^2. Larvæ used for hosts and donors were always chosen from the later "crop" in a bottle.

Spineless Disks in Mutant and Wild Type Hosts

The ss disks developed autonomously without modifying the phenotype of the host flies, H^2 ext, ss, y sc, sc, f and sn^3. No change in size of bristles was found on implants in wild type hosts, or any mutant hosts. Since the bristles in ss are smaller than in +, they are subjected to less compression in the implant. Therefore it may be stated with certainty that development is autonomous in every case. In all the ss implants examined but two abnormal bristles were found, and both of those were split from tip to base. Never were typical forked or singed bristles present.

Hairless Extreme Disks ^{2} in Mutant and Wild Type Hosts

Dorsal mesothoracic disks from H^2 ext stocks were implanted into ss, y sc, sc, f, sn^3 and + hosts without modifying the phenotype of the host. In every case trichopores were identifiable in the implant. In about 50 per cent of the implants some bristles were present, as would be expected.

Statistical treatment of further data would be required to determine whether the average bristle number is (*a*) less in H^2 implants in sc hosts and (*b*) greater in H^2 implants in + hosts, than in H^2 controls. In the first instance, genetic data would lead one to expect an intensification of bristle loss, for when H^2 is crossed to sc the total number of bristles missing is greater than the sum of those removed by the H^2 and sc genes (Sturtevant and Schultz, 1931).

Scute Disks in Mutant and Wild Type Hosts

This series of experiments was carried out with a thoroughly inbred sc stock in which a scutellar bristle is rarely found. Development of sc implants is autonomous in ss, sn^3, H^2 ext, f, y sc and + hosts. No scutellar bristles were found on any implants in this series. Host flies were normal with two exceptions which without further data cannot be considered significant. One wild type male host lacked an upper humeral and an ocellar bristle, while the left anterior scutellar was missing in one wild type female.

Yellow Scute Disks in Mutant and Wild Type Hosts

The development of y sc disks is autonomous in H^2 ext, ss, sn^3, f, sc and + hosts with respect to y character. This is also true of the sc character within the limits of variability which occur in the y sc stock. No modification of the host was observed. These results on yellow body color confirm those of Medvedev (1937) and show that yellow bristle color is also autonomous.

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Forked Disks in Mutant and Wild Type Hosts

Development of f disks is autonomous in H^2 ext, ss, sc, y sc, sn^3 and + hosts without modification of the host phenotype. The f implants can readily be distinguished from sn^3 as the bristles are angular in f and curved in sn^3, although there is considerable overlapping of types (Howland and Glancy, 1937). In the particular f stock used, the microchætæ are straight, while in the sn^3 stock they are singed.

Singed Disks in Mutant and Wild Type Hosts

In every case development of sn^3 disks is autonomous, *i.e.*, in H^2 ext, ss, y sc, sc, f and + hosts, and the host flies are normal. The positive identification of sn^3 offers no difficulty since all the microchætæ as well as the macrochætæ show this character.

Injections of Mutant and Wild Type Body Fluid

Essentially the same series of experiments as those just reported were performed with the exception that injections of body fluid were made instead of imaginal disk transfers. In addition a wider range of ages of both host and donor was employed covering the late larval, prepupal and early (up to thirty-six hours) pupal periods. The injection combinations were made as follows: (1) prepupal fluid into mature larvæ and prepupæ, (2) pupal fluid into mature larvæ, prepupæ and pupæ. Larval fluid was not used.

In many instances the imagoes did not emerge, but had to be removed from the pupa cases. In some of these flies a few abnormal bristles, generally including one or more of the scutellars, were noticed. However, these results can be accounted for on the basis of compression of the developing bristles (Howland and Glancy, 1937). The genotype of the host from which the fluid was taken apparently did not influence the production of these abnormal bristles, for experimental control wild type animals exhibited similar anomalies.

Conclusions

Although there has been no indication of the presence of diffusible substances in ss, H^2 ext, sc, y sc, sn^3, f and + under the experimental conditions described, positive evidence might be forthcoming if (*a*) the entire range of time relationship between host and donor were tested, (*b*) if the amount of injected material were greatly increased or (*c*) if the implanted material were lodged in close proximity to the macrochætæ-bearing regions. The possibility that imaginal bristle-bearing tissues are impermeable to such substances or that they may be unable to release them into the body fluid also deserves special consideration.

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THE EFFECT OF LIGHT ON THE VERTICAL MOVEMENTS OF ACARTIA CLAUSI (GIESBRECHT)[1]

W. H. JOHNSON

(*From the Atlantic Biological Station at St. Andrews and the Woods Hole Oceanographic Institution, Woods Hole, Mass.*)

The changing vertical distribution of plankton Crustacea has long received a great deal of attention; copepods in particular have been the object of much study. Although many factors have been proposed to account for vertical migrations, it is now generally recognized that light is the chief instigator. The complexity of the affair, however, has been frequently discussed by such outstanding authors on the subject as F. S. Russell of Plymouth and G. L. Clarke of Woods Hole. The apparent lack of persistent responses makes it highly desirable to collect more facts dealing with the rôle played by light and it was with this end in view that the following studies were made on the marine copepod, *Acartia clausi.*

FIELD STUDIES

Field studies were carried out during the summers of 1935 and 1936, the collections of the first summer being confined to the head (Big Bay) while those of the second summer centered close to the mouth (off Bain's Island) of Inner Passamaquoddy Bay.

Methods

The two stations were established in regions devoid of any strong tidal currents which might complicate the results. The vertical distributions were determined under various conditions of light by collecting samples at four different depths for each condition of light.

The nets were made from No. 5 bolting cloth. By a special contrivance, it was possible to collect the organisms only at the specific depths; no such device was necessary for the surface tow. The arrangement for opening and closing the nets is shown in Fig. 1. The net is lowered to the specific depth by means of the line "a" which is kept taut, while line "b" runs out freely. In this way the net descends vertically due to the weight "w." On reaching the desired depth, a messenger is let down line "b" thereby releasing the attached mechanism. The net thus takes up the second position and line

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[1] Contribution No. 183 from the Woods Hole Oceanographic Institution.

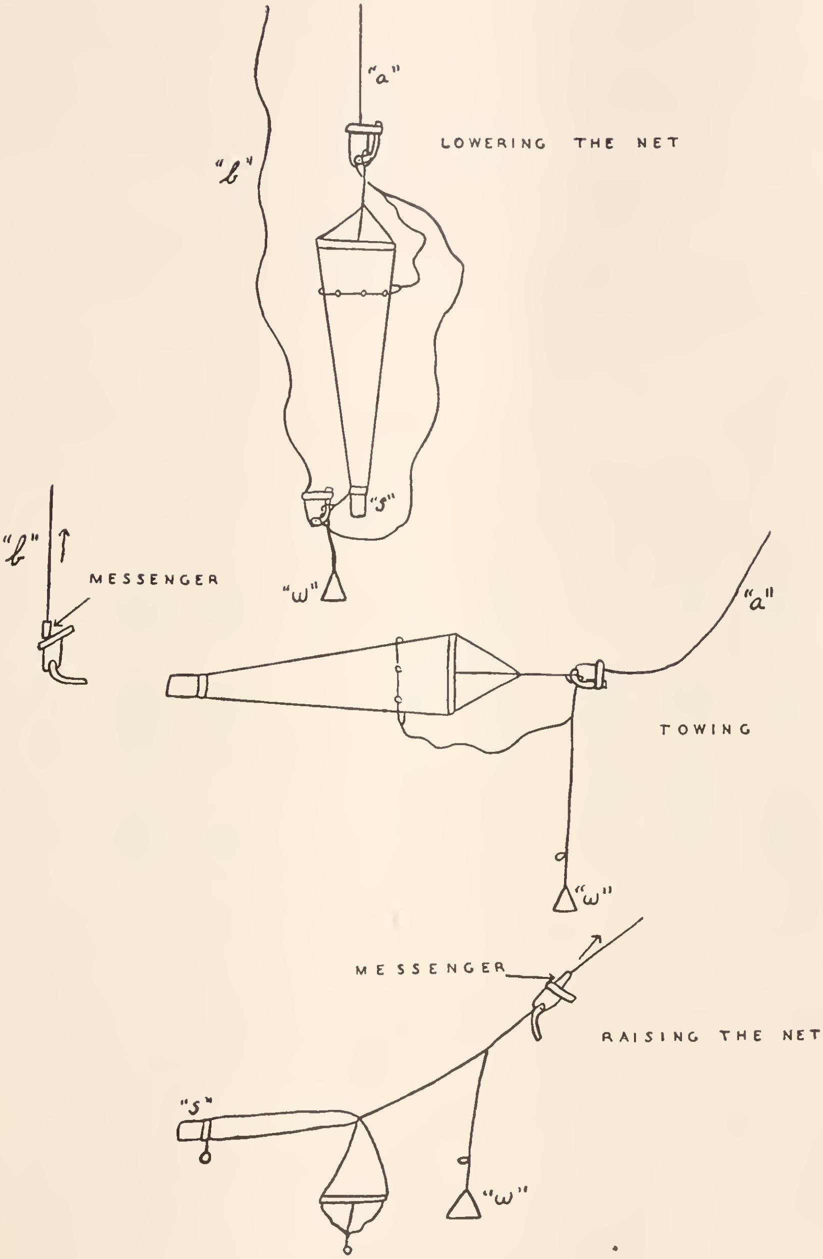
"a"
LOWERING THE NET
"b"
"s"
"w"
"b"
MESSENGER
"a"
TOWING
"w"
MESSENGER
RAISING THE NET
"s"
"w"

FIG. 1. *Closing mechanism used with the nets.*

"b" is withdrawn into the boat. The boat now slowly moves forward causing the net to strain the water. As nearly as can be judged, a definite and constant velocity of the boat is maintained. At the end of ten minutes a messenger is let down line "a" causing the net to be closed and the whole arrangement is withdrawn into the boat. The contents of the net are then carefully washed into the pint sealer "s," preserved in 4 per cent formaldehyde, and labelled.

Just before the nets were towed, the light intensity incident at the surface was measured by means of a solarimeter. This instrument is made by Kipp and Zonen, Delft, Holland. The light-receiving unit is a Moll surface thermopile covered with a hemispherical quartz window. The current produced is measured by means of a micro-ammeter. On a clear day this instrument will measure the intensity of sunlight from sunrise to sunset. The units of light intensity recorded are percentages of full noon June sunlight (F.N.J.S.), i.e. in percentage of the highest reading of noon June sunlight.

The transparency of the water was measured by means of a Secchi disk. It always disappeared from view at 16 feet.

The contents of the pint sealers were treated in the laboratory as follows: permitted to settle in a graduate to determine total volume; thoroughly shaken so as to produce a homogeneous mixture, and exactly 5 cc. removed by means of a Stempel pipette; the 5 cc. sample placed on a specially constructed counting cell, and the copepods identified and counted. At least two counts were made, except when there was so little plankton present that the whole tow was counted without making use of the Stempel pipette. The results of the counts were averaged. If the counts were within 10 per cent of their average, no further counts were made. The counts, when specimens were numerous, always showed close agreement. It was, however, often found necessary to make as many as six counts of the scarcer forms.

Each copepod was determined as being male, female, or young. For the purpose of identification, the following authors were consulted: (*a*) Sars—"Crustacea of Norway," and (*b*) Wilson—"Copepods of the Woods Hole Region." Such identification was not difficult as no very young forms were present in the tows, and the young found were sufficiently like the adults not to be easily confused as to species. The size, shape of fifth leg, antennule, and number of segments of the urosome were the characters mostly used for identification. The counting cell was so shallow that the copepods usually lay on their sides, thus exposing the fifth leg. A drop of dilute aqueous solution of methylene blue, added to the contents of the counting cell, greatly facilitated counting of the segments and recognition of the shape of the fifth legs.

Results

Consecutive Samples:—The results of a 24-hour series taken off Bain's Island on September 2 and 3, 1936, are shown in Fig. 2.

During the morning of September 2, the sunlight intensity was increasing rapidly so as to reach 60 per cent F N.J.S. by 10 A.M. Males, females, and young were all absent from the surface with the young, on the whole, not as far down as the adults.

By 2 P.M. the intensity had increased to a maximum of 78 per cent F.N.J.S. Occasional clouds in the sky, however, cut the intensity down to about 28 per cent F.N.J.S. when crossing before the sun. However, the prodominant light intensity was the maximum value recorded, and it is seen that males, females, and young are now in proportionately greater numbers at the bottom than before.

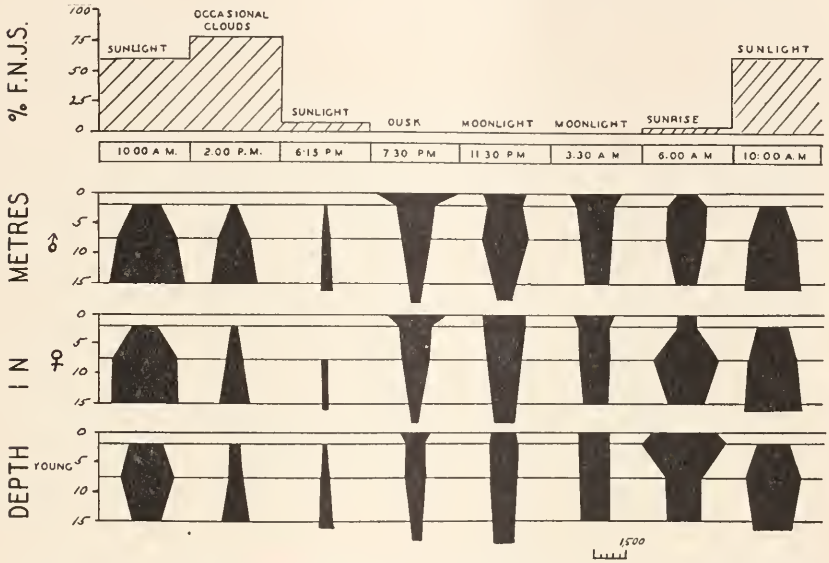

FIG. 2. The vertical distributions of males, females, and young over a period of twenty-four consecutive hours in September of 1936.

During the late afternoon, the sunlight was decreasing rapidly so that at 6:15 P.M. the light was 7 per cent F.N.J.S. Unfortunately, low numbers were captured by the nets at the time and some justification is felt for believing that the true picture of vertical distribution is not presented here.

A considerable drop in intensity occurred in the late evening so that by 7:30 P.M. a condition considered as dusk prevailed. The photometer would not register at this low intensity; the visibility was very poor. During these conditions it is quite evident that a decided upward migration for males, females, and young had taken place so that decided maxima now occurred directly at the surface.

Shortly after 7:30 P.M. the full moon had appeared over the hills and by 11:30 P.M. visibility was good. Thus there was an increase in light intensity from 7:30 to 11:30 P.M. and on comparing the distributions at these two times males, females, and young alike appear to show a downward migration from the surface. It may be, however, that the moonlight had no effect and the animals were becoming uniformly distributed.

Moonlight prevailed throughout the rest of the night till 5:00 A.M. and tows taken at 3:30 A.M. indicate a decided tendency for uniform distribution of males, females and young. Thus continued moonlight does not result in their concentrating at any one particular depth.

At 6 A.M. the sun had risen over the hills causing an increase in light intensity as compared to the condition during the previous night. Males, females, and young all showed a downward migration from the surface with the young least affected, then the males, and finally the females. That is, the young showed a maximum at 2 meters, the males from 2 to 7½ meters, and the females at 7½ meters.

The sunlight continued to increase in intensity until by 10 A.M. it had reached 60 per cent F.N.J.S. Males, females, and young had descended still deeper into the water as compared with the condition at 6 A.M., with the males and females now showing maxima at the bottom and the young at 7½ meters. The young were still less affected by this condition than the adults. The vertical distributions are similar to those which obtained the previous day at 10 A.M. when the same light conditions prevailed.

Another series of consecutive tows was taken during the summer of 1935, the results of which are given in Fig. 3. During the late afternoon, the sunlight intensity was decreasing from 75 per cent at 2:40 P.M. to 27 per cent F.N.J.S. at 5:40 P.M. The two-meter tow taken at 2:40 P.M. was unfortunately destroyed. Nevertheless, it is evident that a decided upward migration had taken place for males, females, and young directly to the surface at 5:40 P.M.

The moon, which was two-thirds full, had been up all evening and so the light intensity was decreasing more slowly than had there been no moon. On comparing the vertical distributions of 8:55 P.M. with those which obtained at 5:40 P.M., it seems that a downward migration was taking place from the surface—possibly the result of the rate of decrease being so low as to no longer attract the animals up to the surface such as occurred somewhat earlier in the evening, i.e. the rate of decrease must have been below their threshold (Clarke, 1930). It might have been, had the light continued as it was at 8:55 P.M., that the animals would later have become uniformily distributed. However, the moon set shortly before midnight so that the condition at 12:10 A.M. represents a decrease in light intensity. Here we have evidence of an upward migration in the upper layers to the surface—a migration particularly evident for the males and young.

During continued starlight conditions, males, females, and young tended to sink slowly in the water as seen by the condition at 3:20 A.M.

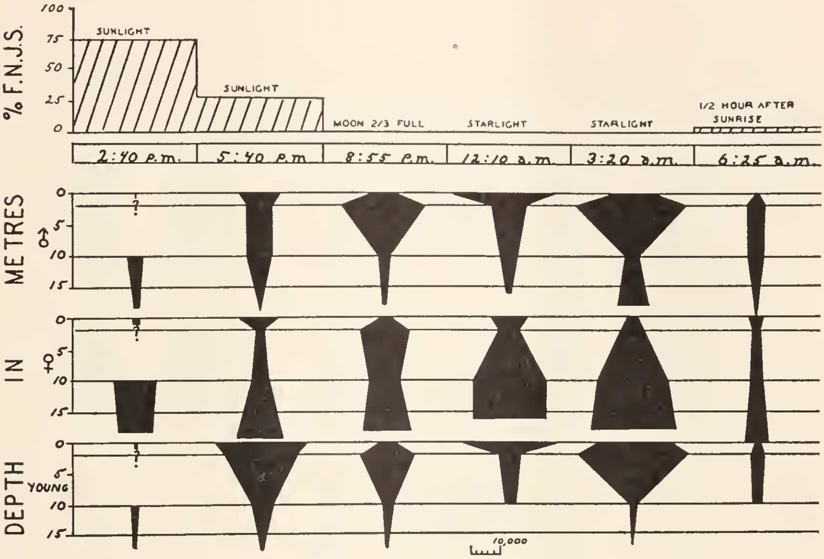

FIG. 3. The vertical distributions of males, females, and young during consecutive hours in August of 1935.

With the sunlight increasing in intensity in the early morning, reaching 3 per cent F.N.J.S. at 6:25 A.M., and comparing the vertical distributions with those at 3:20 A.M., it is quite evident that a great decrease in numbers had taken place in the upper levels. Although the animals appear to be more or less uniformly distributed, it is believed that a downward migration is in the process of being carried out, a fact which would have been made more evident had the tows been taken somewhat later (cf. Fig. 2 at 10 A.M.).

Occasional Samples:—From the above results it is seen that upward migration occurs under decreasing light, while downward migration occurs under increasing light. Although similar vertical distributions were found to occur on different days when the light intensities were the same, it soon became evident that this is not always the case. Thus at sunrise on August 1 (Fig. 4), the animals were all farther down than at sunrise on September 3 (Fig. 2) even though the light intensity was the same on each occasion. However, the light intensities preceding sunrise were different on the two occasions; in the former case there had been no moonlight from 2:30 A.M. on, while in the latter case there had been full moonlight all night until one-half hour before sunrise. In other words, following the greater increase in light intensity the animals descended farther down.

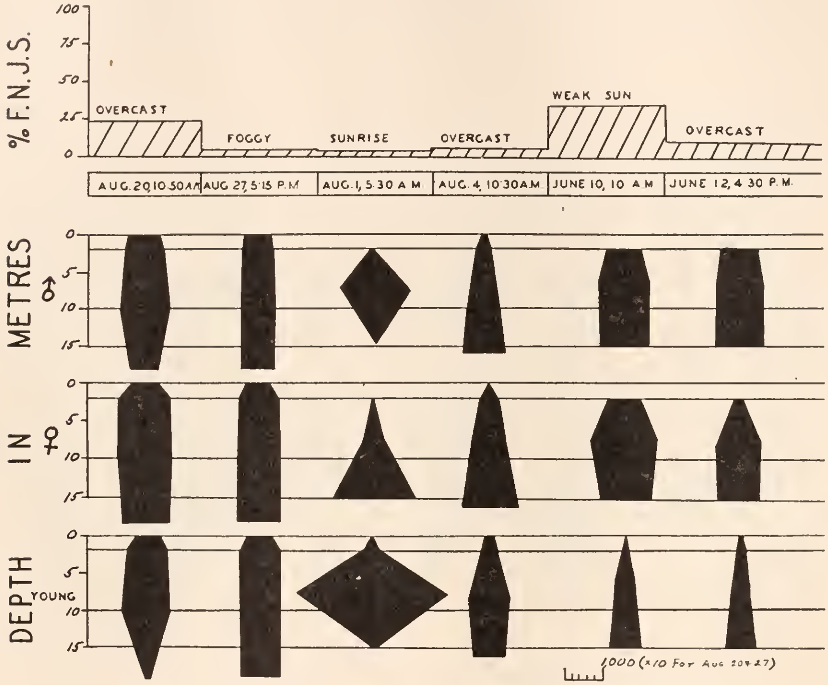

FIG. 4. The vertical distributions of males, females, and young as determined by occasional tows.

More examples indicating the difficulty of explaining vertical distributions on a basis of optimum intensity were found. For example, on June 12 at 4:30 P.M. males, females, and young showed practically the same vertical distributions as on June 10 at 10 A.M. (Fig. 4) even though the light intensities were decidedly different on the two occasions (10 per cent and 35 per cent F.N.J.S. respectively).

Increasing light apparently drives the animals down from the surface. When the light is steadily increasing in the morning, the higher the light intensity the farther down the animals are found (as has already been shown). However, this relationship between light intensity and depth does not necessarily hold when we compare the vertical distributions at the same time of the morning on two separate days having different intensities. This is well shown in Fig. 4. On August 20 at 10:50 A.M. males, females, and young were all, on the whole, nearer to the surface than at 10:30 A.M. on August 4, even though the light intensity was much higher on the former occasion (25 per cent F.N.J.S. as contrasted with 5 per cent F.N.J.S.).

LABORATORY EXPERIMENTS

Laboratory experiments, the results of which might prove to be of value in attempting to interpret the behavior of *Acartia clausi* in nature, were carried out in the basement of the Atlantic Biological Station during the latter part of September, 1936.

We have seen cases where reliable results showed that similar vertical distributions can take place under different light intensities. This fact was noted for males, females, and young alike of the same species and in the same locality. If the animals change their vertical distributions with changes of light intensity, is the direction of migration such as to bring them into a region of the same light intensity as that in which they existed formerly? Or, in other words, is there an optimum intensity for which the species is continually seeking? The very fact that similar vertical distributions can be found under different light intensities may answer the question in the negative in certain cases. However, to test the question somewhat more extensively, it was decided to study their vertical distributions under controlled conditions of light in the laboratory.

Methods and Results

Two tows were made off the Atlantic Biological Station wharf at St. Andrews at 4 P.M. during bright sunlight of intensity 50 per cent F.N.J.S. at the surface. The tows were made at the surface catching a few hundred *Acartia clausi*. Very few of any other species were present, *Acartia* being dominant in this region. In a quiet region it would be difficult to obtain *Acartia* at the surface under such conditions, but due to swift tidal currents they are forced up to the surface here.

Each tow was placed in a separate glass cylindrical glass jar of about 20 inches in length and 5 inches in internal diameter. Tar paper was placed around and below each jar so that light could only come in from the top. A piece of thin glass was placed just above the open upper end of each jar so as to absorb heat from the electric light bulb when the latter was close to the jar. Over each jar was placed an electric light bulb of 50 watts, capable of being raised or lowered through a distance of 5 feet above the surface of the water in each jar. The arrangement was set up in a constant temperature room. The temperature of the water in the jars was thus kept the same as the water in nature from which the copepods had been removed (12° C.).

Effects of Single Changes in Intensity

Over one jar the electric light bulb was placed at a distance of 5 feet from the surface, while over the other a bulb was fixed a foot from the surface. The animals which had been obtained from the surface under bright sunlight were allowed ten minutes to distribute themselves and then the vertical distributions were determined by counting the numbers seen during a period of one minute through a square hole (1/4 inch square) in the tar paper. Such a count was made for each quarter-length of the jar. The resulting vertical distributions are shown in the following table:

	Bulb at 1 foot	Bulb at 5 feet
Section I (upper)	22	60
Section II	16	2
Section III	16	2
Section IV (lower)	1	2

The animals were subjected, as a result of the above procedure, to a decrease in light intensity in each case (sunlight intensity at the surface in nature was 50 per cent F.N.J.S., while a 50-watt bulb, when placed at a distance of one foot from the photometer, produced a reading of 4 per cent F.N.J.S., the intensity at a distance of 5 feet being 0.18 per cent F.N.J.S.). It is seen from the above that both changes in light intensity resulted in upward movement the extent of which was greater following the larger decrease in intensity. The individuals were uniformly distributed in the jars before being taken into the laboratory.

The electric bulbs were then left in the above positions until 2:30 P.M. the next day, and on examining the vertical distributions it was found that the individuals were uniformly distributed in each jar. The results point to a decrease in light intensity as the stimulus for upward migration and the greater the change in light the greater the upward movement. Furthermore, they indicate that the animals were not seeking an optimum intensity since they became uniformly distributed when left for some time under the intensity in which they first moved upward.

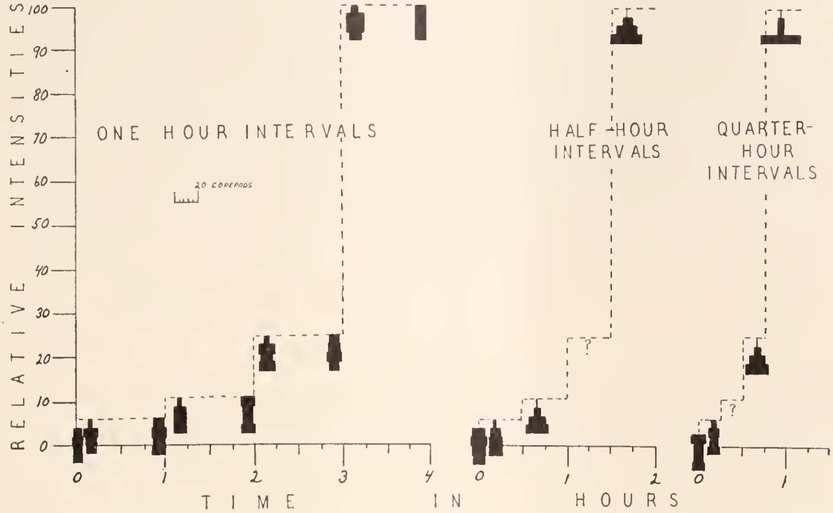

FIG. 5. The vertical distributions in glass jars resulting from increasing the light intensities at different frequencies.

The positions of the bulbs were then reversed so that the bulb previously at the surface was raised to 5 feet causing a decided decrease in light intensity, while the bulb previously at 5 feet was lowered to 1 foot from the surface causing an increase in light intensity. The vertical distributions ten minutes after the changes were made are shown in the following table:

	Bulb at 5 feet	Bulb at 1 foot
Section I (upper)	28	1
Section II	14	4
Section III	5	14
Section IV (lower)	1	30

The results show that a decrease in light intensity resulted in upward movement while an increase in light induced downward movement. The magnitudes of increase and decrease in both cases were the same and it is interesting to note that the resulting vertical distribution of the one is practically the inverse of the other. The bulbs were left this way until 9 A.M. the following morning and on examination a uniform vertical distribution was found in each jar. Thus it again appears that a change in light intensity is necessary to evoke migration of *Acartia*, while under static conditions no movement occurs.

Effects of Repeated Changes in Intensity

Increasing Light:—With the animals uniformly distributed and the bulb at 5 feet, the light intensity was increased by lowering the bulb 1 foot every hour, 1 foot every half-hour, and one foot every 15 minutes. The vertical distributions were determined shortly after each change was made, and in the case of one-hour intervals, readings were also made just before the next change. The results are shown in Fig. 5.

Decreasing Light:—With the animals uniformly distributed and the bulb at 1 foot, the light intensity was decreased by raising the bulb one foot every hour, one foot every half-hour, and one foot every 15 minutes. Readings were taken of the vertical distributions 10 minutes after each change was made, and in the case of one-hour intervals, readings were also made just before the next change. The results are shown in Fig. 6.

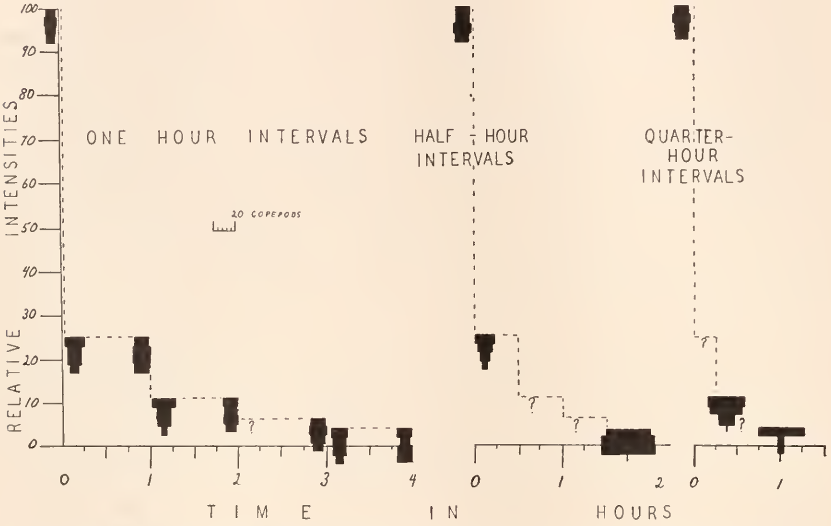

Fig. 6.—The vertical distributions in glass jars resulting from decreasing the light intensities at different frequencies.

It is seen from the above two experiments, that on increasing or decreasing the light intensity by lowering or raising the bulb one foot every hour, slight but noticeable changes were produced in the vertical distributions of the copepods soon after each change was made, but that the animals returned to uniform vertical distribution before the next change was made. In the case of the half-hour intervals, the animals did not return to uniform vertical distribution but gradually increased or decreased the distance at which the majority were found from the surface; the same applies to the 15-minute intervals except that the movements were more pronounced.

It is thus seen that when the light intensity remained the same for some time before the next change was made, the animals returned to uniform vertical distribution; but if the next change followed soon enough the animals continued to move either up or down, as the case may be, and the more frequent the changes the more pronounced the effect.

Examples are also seen where similar vertical distributions occurred under different light intensities, and also examples of different vertical distributions under similar light intensities.

Discussion

While the vertical distributions of *Acartia clausi* in nature were found to present some puzzling problems, it appears that some importance might be placed upon the direction and rapidity of change of light intensity, i.e., downward migration may be brought about by increasing light intensity while upward migration may be brought about by decreasing light, and some instances were found which suggest that the more rapid the change of intensity, the greater the response induced.

It is realized that the changes of light intensity produced in the experiment do not represent different rates of change but were merely different frequencies of change, and each change in itself was more sudden than usually occurs in nature (except possibly the sudden increase and decrease of light as the result of a cloud passing across the sun). Furthermore, it is realized that during any one series of changes the different magnitudes of change were in no two cases equal; thus the experiment does not represent the effects of similar magnitudes of change merely being applied at different frequencies. Taking everything into consideration, the results of the experiment are not strictly applicable to light changes as they usually occur in nature. Nevertheless, they do suggest that *Acartia clausi* requires *changes* in intensity to evoke vertical migration and the more frequently the changes occur the more the animals are stimulated to move either toward or away from the light as the case may be.

An interesting fact not already mentioned is that *Acartia* moved toward an electric light (or bright sunlight) after having been in darkness, while, as was shown above, they moved away from the bulb when the latter was brought closer to the animals after they had been previously in the light. Similar responses have been noted by L. B. Clark (1931) for the insect *Dineutes*.

Although it is felt that the facts presented in this paper by no means solve the manner in which light controls the vertical movements of copepods in the sea, it is believed that they are worth while considering before the very complicated problem of phototropism be solved.

ACKNOWLEDGMENTS

The writer wishes to express his gratitude to Dr. A. G. Huntsman of the Biological Board of Canada for stimulating the investigation in many ways; also to the Biological Board of Canada, the Department of Biology of the University of Toronto, and the Woods Hole Oceanographic Institution, under whose auspices the investigation was carried out.

For criticism of the manuscript, the author feels indebted to Dr. A. G. Huntsman of the University of Toronto, and to Dr. G. L. Clarke of Harvard University and of the Woods Hole Oceanographic Institution.

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ON THE EARLY DEVELOPMENT—BIPOLAR DIFFERENTIATION AND CLEAVAGE—OF THE ZEBRA FISH, BRACHYDANIO RERIO

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(*From the Biological Department, Brown University*)

Introduction

Extensive studies of the early development of teleosts are very rare. The separation of the protoplasm from yolk and the first cleavages have been most thoroughly observed by Kowalewski (1886). Spek (1933) was the first to solve some of the problems connected with bipolar differentiation, especially from the point of view of colloid chemistry. The few morphological descriptions of the first events are for the most part simply qualitative. In only one paper, of His (1876), can exact measurements and a series of continuous quantitative observations be found. These, however, are little noticed in the literature, since they are reported as individual items without adequate statistical analysis.

The development of *Brachydanio rerio*, the zebra fish, a cyprinid from India, has never been described, and has been experimentally treated, as far as I know, only incidentally in a work of J. Oppenheimer (1936*d*), in which nothing is mentioned about the normal development of the egg. Of all the fishes whose development has been described *Cyprinus blicca* seems most like the zebra fish (v. Baer, 1835). From v. Baer's pictures it appears that the proportions of yolk and protoplasm and also the rate of development are almost the same. His discussion, however, begins with the blastula stage.

In some respects, *B. rerio* is an extraordinary fit laboratory object. Eggs in considerable number can be obtained every day of the year. The egg is transparent, has a small yolk and can be held in any desired position without trouble. Furthermore, the development is unusually rapid. The time from fertilization to hatching is about 60 hours at 27° C. Since the development presents several differences from the species already described and since so little is known about the earliest development of teleosts, it seems worthwhile to publish a simple description as a basis for further experimental study.

Material and Methods

The animals were kept in tanks of about 60 cm. length and 40 cm.

width with a water level of at the utmost 25 cm. In a corner, the bottom was covered with stones of 2–6 cm. in diameter, over which a dense clump of plants was placed. The most successful and probably most natural propagation will be obtained by keeping the fishes in schools of 12–15 specimens, of which 8–10 should be males to guarantee abundant fertilization. The most fertile age is from ¾ to 1½ years. The optimal temperature is 26–27° C., most conveniently held by a thermostat. If uniformly healthy eggs are desired, it is necessary to have an abundant artificial aeration and even circulation of the water. Under such conditions the zebra fishes spawn almost every day, but the same individuals do not always take part, particularly not the females. The spawning occurs in the early morning at about 8 o'clock in winter, in summer often as early as half past five. The fertilized eggs drop into the plants and among the stones. The parents are not able to get the eggs to eat them, though they try continuously to find them.

The eggs were caught with a dip-tube. Occasionally I fished them up while they were sinking and was thus able to observe the very early stages. Usually they were pipetted into small dishes, which were wrapped in gauze and placed in the aquarium near the aerating equipment. It is easy to obtain 10–50 eggs daily during the first 3–4 weeks of spawning. Later the number decreases and there occur intervals of 1–4 days. With copious and varied food, research material is available almost continuously, as the young ones spawn at 5–7 months of age and the seasons have practically no influence on the fertility in the aquarium.

OBSERVATIONS

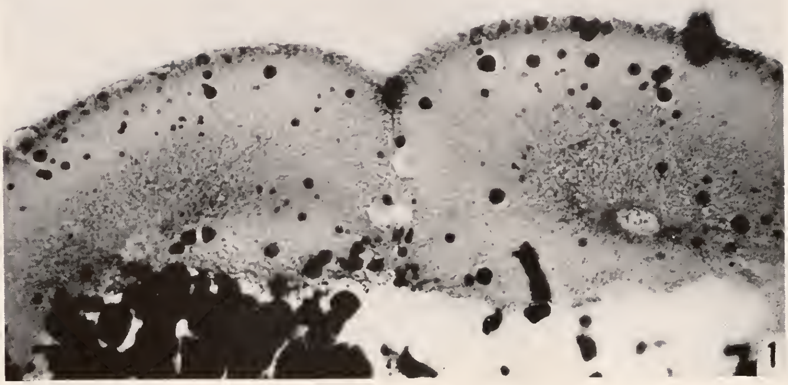
Most observations were made on the living egg. Unless especially designated as averages, the measurements in this paper are all taken from two single eggs, the development of which has been recorded in a film. This film was taken with time accelerator under the microscope, and a partial description of it has already been published (Roosen-Runge, 1936). It is necessary to emphasize that the greatest advantage of such a film for science is not in looking at it, projected on the screen, though that may show an interesting survey. Careful measurements combined with comparison of the running film taught me to appreciate the film as a research method. Especially for the rapid events before the first cleavage measurements gave much better information than the observation of the running pictures. I used a copy of small size (16 mm.) from the standard sized negative and measured picture by picture with an eye-piece micrometer under the low power of the microscope. For the most important parts I had pictures of two different eggs to work with. While taking the film the eggs developed under a constant temperature of 27° C. The pictures were done every 1½ second corresponding to a 24 times acceleration. The film was financed and assisted by the Deutsche Gesellschaft für wissenschaftliche Filme m.b.H., Berlin.

THE DEVELOPMENT

The following description will deal only with the development of the egg after fertilization. The mature egg of *B. rerio* is white and nearly opaque, homogeneously filled with granules. The average diameter is about 625 μ. When the egg leaves the oviduct it is apparently not so completely differentiated as the eggs of most teleosts. For example, in *Corregonus macrophtalmus, Salmo irideus, Squalius cephalus, Leuciscus rutilus, Barbus fluviatalis, Tinca fluviatalis* and *Gasterosteus aculeatus*, it was observed by Spek (1933), that a hydration of the cell substances has already taken place in the ovary, producing a much more transparent appearance in the eggs, and that in all these species bipolar differentiation is obvious before fertilization occurs. In the ripe egg of *B. rerio* there is no visible separation of the blastodisc. This does not mean that no differentiation exists. The polarity and the later bipolar differentiation is foreshadowed by the location of the nucleus and the very small amount of protoplasm around it. When the egg is laid, the water causes only a slight concentration of the protoplasm. This had been previously observed in the herring (Kupffer) and in the perch and the pike (Lereboullet), but most teleost eggs show in the ovary a distinct blastodisc, the increase in size of which is strongly accelerated by the contact with water.

As I was never successful with artificial fertilization, I cannot report anything about the first minute of development from direct observation. My earliest observation begins about a minute after fertilization. The film, for which the egg had to be brought into position, does not start before 1½ minutes, and so the exact measurement does not begin earlier. During the first minute the egg is already changing rapidly. Due to the fact that it is not completely differentiated in the ovary, all the first events are condensed into a very short time, so that during the first 15 minutes many more things happen than during the first 15 hours in the development of the trout (this is in both cases until the time of the first cleavage).

Before entering into a detailed description of these early events it is necessary to say something about the method of orienting the egg. There is a polar axis in the mature egg of the zebra fish just as in



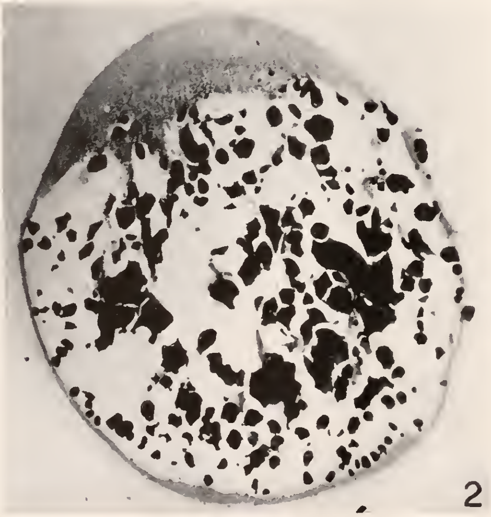


FIG. 1. Section through two cells of a 4-cell-stage. Nuclei in early prophase. Stained with Heidenhain's hematoxylin. 8 μ. × 810.

FIG. 2. Section through an egg during early separation process. Yolk broken. The protoplasmic coat is visible around the cell, with a slight concentration at the vegetal pole. Stained with Heidenhain's hematoxylin. 8 μ. × 115.

other teleosts, though it is less conspicuous. Immediately after the membrane is lifted off (Fig. 1), the egg appears somewhat oval in shape with the long axis as the polar axis and the animal pole marked by a little depression, where the sperm entered. But it is far more important for the orientation of the egg that, in addition to this, the direction of the first cleavage plane seems also to be established very early. The cavity on the animal pole is from the first moment of observation slightly elongated, so that it appears deepest when seen along the long axis. It is therefore possible to orient the egg during the earliest stages by turning it around the polar axis, until the cavity appears most excavated. If this is done (Fig. 1 in this paper, Fig. 1*a* in Roosen-Runge, 1936) it is almost certain that the first cleavage plane will appear perpendicular to the plane of observation. Figure 7 shows an egg in the fifteenth minute that had been exactly oriented in the described way. In Fig. 11 the first cleavage of the same egg can be seen. The direction of the first cleavage plane can be foretold at least shortly after fertilization. It remains a question whether or not it is established by the direction of the sperm entrance. If so, the determination must take place in the very short time of about one minute.

About one minute after fertilization the egg has a diameter of about 750 μ from membrane to membrane. It is not quite round but the deviations are usually small. The membrane is soft and very extensible. Spek discovered that in teleosts it consists of the primary membrane of the oöcyte and the radially striped cortical layer clinging to it. The external protoplasmic layer of the oöcyte takes up water and lacunæ appear, which later flow together and establish the perivitelline space. Still later there begins an enormous secondary enlargement of the space, because water has been attracted into it. But in *B. rerio* some material for this enlargement evidently comes from the egg itself during the first few minutes, since the cell shrinks at first, though the egg as a whole is swelling. After the second minute the cell is about 580 μ in the equatorial diameter and 605 μ in the polar diameter. One and a half minutes later (Fig. 2) the equatorial diameter is still the same, 580 μ, but the polar diameter has decreased to 565 μ, which means that together with a pronounced change in shape, the egg shrank and lost material into the space. The shrinking of the cell takes only a very short time of about 3½ minutes. At the end the cell appears more round (Fig. 2) but the pole of the faintly visible blastodisc is somewhat more flattened. The diameter from membrane to membrane has increased from 750 μ to 830 μ. During the following 2 to 3 minutes the cell itself increases greatly in volume, probably due to an increased hydration of its substances.

Plates I and II

Explanation of Figures

Photomicrographs of living zebra fish eggs, taken from a film. Development at 27° C. × 19.

Figs. 1–6 present one egg in the different stages. The egg is oriented so that the first cleavage plane will occur perpendicular to the picture plane. The egg turned between Figs. 5 and 6, so that only a small part of the blastodisc is visible in Fig. 6.

Figs. 7–23 present another egg. The orientation is exactly the same as in Figs. 1–5

PLATE I

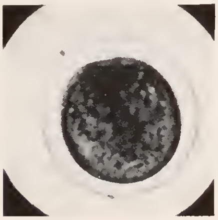


Fig. 1. 2 min.

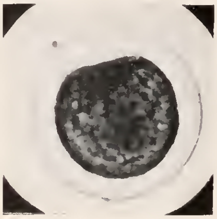


Fig. 2. 3½ min.

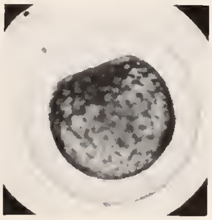


Fig. 3. 4½ min.

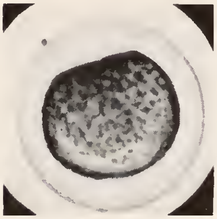


Fig. 4. 6 min.

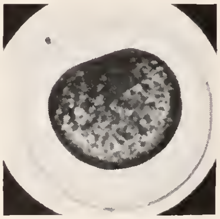


Fig. 5. 9 min.

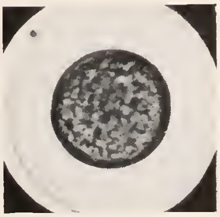


Fig. 6. 15 min.

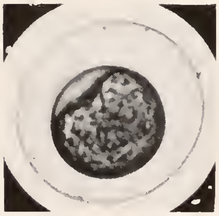


Fig. 7. 15 min.

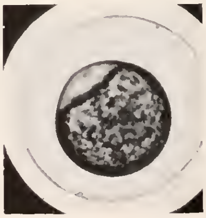


Fig. 8. 19 min.

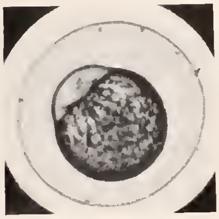


Fig. 9. 22 min.

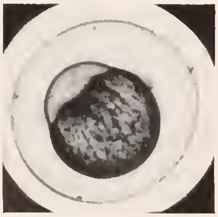


Fig. 10. 24 min.

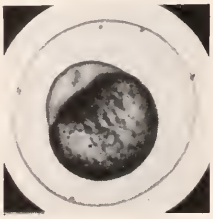


Fig. 11. 32 min.

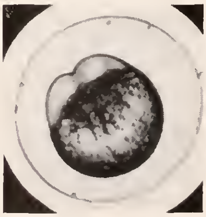


Fig. 12. 40 min.

In describing the following change in shape the diameter of the first cleavage plane is referred to as the frontal diameter; the sagittal diameter is that of the second cleavage plane. These terms have no reference to the future planes of symmetry of the embryo. The longest diameter is always used. The stage in Fig. 4 has a polar diameter of 575 μ but a sagittal diameter of 665 μ. At this time the sagittal diameter is 15–25 μ longer than the frontal diameter. The egg is somewhat elongated, as though it were foreshadowing the direction of the spindle axis of the first cleavage which is to follow. The elongated shape disappears entirely from the fifteenth minute until it reappears about the thirtieth minute at the time of the first cleavage. An elongation is observed as the first cleavage spindle develops in most eggs, but the previous lengthening is unusual and the relation of the two is not clear at present.

At about the fifth minute the change of the cell substances is very conspicuous. The cell becomes more transparent in a short time beginning at the vegetal pole. If mixed with water the substances coagulate in a much finer and less granular form than if such a mixture is made during the first minutes. They are probably changed in their colloidal condition by a strong hydration. The enlargement of the cell is greatest at exactly the time when the animal pole is most flattened, and from exact measurement it can be seen that the increase in volume of the whole egg, that is the taking up of water through the membrane, begins to slow down at the same moment. It can be seen in Fig. 4 that there is a very thin, clear protoplasmic coat around the cell in direct connection with the blastodisc. This coat has gradually appeared during the preceding minutes. It shows an uneven outer surface which changes its shape continuously. The movements of the protoplasm are comparable to the so-called amœboid movements, observed in the trout shortly after fertilization (Oellacher, 1872). The protoplasmic layer is very distinct for 2 to 3 minutes and then begins to disappear with the next changes of the egg until it is entirely invisible in the ninth minute (Fig. 5). The cell rounds more and more and the blastodisc, flattened to a thin and even layer, becomes also round and higher with a smaller base. There is a conspicuous enlargement of the blastodisc now for the first time. Not the slightest streaming which would indicate a separation of protoplasm from yolk is visible, but the protoplasmic cover has become so fine that it is traceable only in the sections. The concentration of protoplasm into the blastodisc, at this time, seems to be an entirely different process, from what will be presented later as the separation of protoplasm from yolk. An actual separation must have happened earlier when the clear protoplasmic layer appeared around the cell. At the time of the rounding of the egg the protoplasm concentrates from this layer into the blastodisc and thins out consequently. This phenomenon is unusual and seems never to have been described.

After the stage in Figs. 6 and 7 is reached, there is an obvious break in the development, which is marked particularly by the fact that no visible changes happen during the next 5 minutes. No movements of yolk substance, nor of particles inside the protoplasm are visible. The cell is exactly round; all diameters are about 605. Shortly before the spherical shape was attained, the swelling of the egg as a whole had ceased, and the diameter of the egg remains constant from now on till the embryo hatches. That means that the volume of the egg does not change any more and that the membrane no longer expands. The egg finally has a diameter of 1 mm.

The first movement inside the yolk is to be seen at about the fifteenth minute after fertilization. At first it appears as a fluctuation in the granular mass without a prevalent direction, but there must be a directed migration of material, since the blastodisc is already growing. The area between yolk and blastodisc, the "Mischzone," which appears as a dark refracting zone in Fig. 7, becomes smaller until after 7 minutes it has almost disappeared. At that time (Fig. 9) conspicuous lines become visible in the yolk, caused by a directed streaming of protoplasm into the blastodisc. The material comes, at first, not so much from the center of the yolk, but flows more vigorously from the superficial parts, particularly from those near the blastodisc. The lines converge towards the center of the blastodisc, where they form acute angles. Two dark areas which appear symmetrically in the blastodisc at the base mark the location of the nuclei. The higher density of the protoplasm around the nuclei can also be observed in the stained sections (Fig. 1 in the text). When the position of the nuclei first becomes evident in the living egg, the darker areas around them are apparently distorted by the streaming of the protoplasm. The view from the animal pole confirms this, showing that the stream comes onto the blastodisc through the central area of the base, then spreads towards the upper part of the cell surface like a fountain. The most superficial layer is not involved in the movement. Granules appear anywhere throughout the cell, except in the immediate vicinity of the nucleus. Some of them lie in the surface layer; they do not take hematoxylin and look faintly yellowish in the sections. These cannot be distinguished from the stainable granules in the living egg.

At about the twenty-second minute the direction of the stream begins to change. The lines in the yolk appear more nearly vertical and very soon almost perpendicular to the base of the blastodisc. At this time the "Mischzone" begins to grow rapidly, reaching its climax when the cell is cleaving. It represents a very dark and wide zone, which is situated under the central region of the blastodisc (Fig. 11). It apparently consists of fine granules mixed with larger yolk drops; between the two substances exists a great difference of refraction (Spek, 1933, p. 522). I was able to observe in the running pictures at this time a distinct stream of numerous fine granules in the protoplasm, apparently in the layers immediately underneath the surface. The direction of this stream is opposite to that of the axial stream in the yolk, but the velocity is about the same. It is visible only against the background of the contrasting yolk drops and not in the blastodisc itself. It seems probable that the concentration of protoplasm at the vegetal pole, which has been observed by Kowalewski (1886) and can be easily seen in the sections of *Brachydanio* eggs at this stage (Fig. 2 in the text), is only a consequence of the counterstream in the protoplasmic coat. The fact of this counterstream is of great importance, particularly with respect to the observations of Spek (1933, p. 524). He never saw any counterstream in teleost eggs and therefore denies that a lowering of the surface tension at the animal pole could account for the streaming of the protoplasm in that direction. I myself, though I knew the fact from the film, was never actually able to see it in the living cell. Only by means of speeding up the movement was it possible to overcome the great difficulty of distinguishing the fine transparent protoplasmic stream from the coarse movement in the yolk.

The first cleavage is indicated by a slight depression at the animal pole at about the thirty-second minute (Fig. 11). A few minutes later the cells are divided by a line but not separated from each other (Fig. 12). The division is almost complete and no protoplasmic connection can be observed at the base of the cells as in most other teleosts, for instance, in the trout. The border can be stained with Heidenhain's hæmatoxylin as a very fine but distinct line (Fig. 1 in the text). As the blastomeres separate, the "Mischzone" between yolk and blastodisc decreases, passing a stage where it seems to consist mostly of large distinct granules (Fig. 13, Plate II). In as much as the nuclei are usually divided at the twenty-second minute, the time which it takes to complete the cleavage seems relatively long. After the cleavage is completed (Fig. 14) the cells lose their spherical shape again and show a wide plane of contact. The streaming of the protoplasm, which ceased after the cells were completely rounded, begins in the parts nearer to the blastodisc as it did the first time and

PLATE II

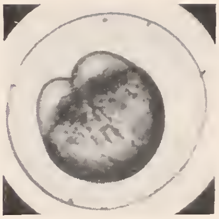


Fig. 13. 47 min.

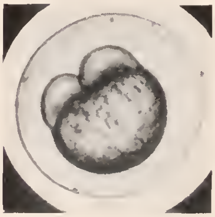


Fig. 14. 51 min.

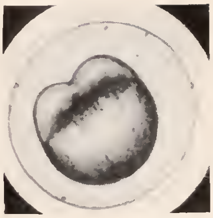


Fig. 15. 65 min.

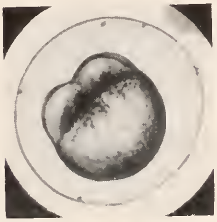


Fig. 16. 67 min.

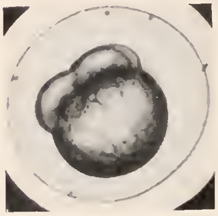


Fig. 17. 80 min.

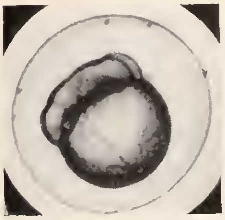


Fig. 18. 92 min.

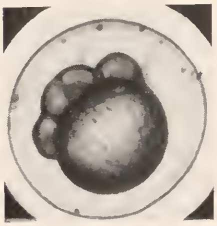


Fig. 19. 104 min.

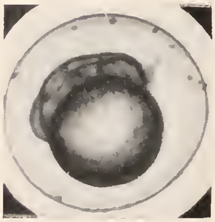


Fig. 20. 117 min.

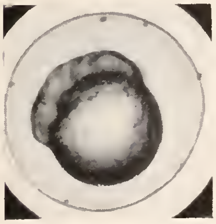


Fig. 21. 128 min.

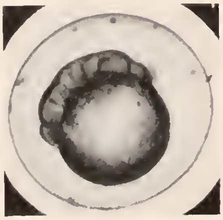


Fig. 22. 136 min.

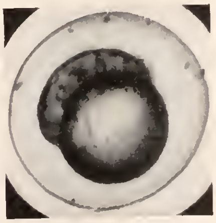


Fig. 23. 146 min.

gradually increases till the stream comes from all parts of the yolk (Figs. 15 and 16), but the stream is not as vigorous, of course, because the amount of protoplasm left in the yolk is very much smaller.

The "Mischzone," which appeared not very voluminous when the first cleavage was completed, becomes more conspicuous again. The climax of the separation process and the formation of the "Mischzone" is reached when the second cleavage plane becomes visible, perpendicular to the first one (Fig. 16). During the further cleavages this cycle can always be seen to a gradually decreasing extent: the cells lose their round shape when the "Mischzone" and the stream into the blastodisc is minimal; they appear adherent to each other with wide contact areas, when the volume of the "Mischzone" and the stream are at their maxima, and at this moment the cleavage plane becomes first visible (Fig. 18). Then the cells round again, become almost separated and the streaming discontinues (Fig. 19). The stage of 16 cells is to be seen in Fig. 20. The cells are rounded, so that three of the four rows of cells, one behind the other, can be distinguished in the picture. The same cells appear flattened as they divide into 32 (Fig. 21). They have rounded again in Fig. 22, which shows the cells on the outside more spherical, because they are not as widely adherent to surrounding cells. At the time of the first tangential cleavage the cells are again more flattened and the outline of the blastodisc appears smooth (Fig. 23).

The first cleavages in *B. rerio* always appear in the same order and show in every respect a considerable constancy. The type of cleavage is the same as in *Fundulus heteroclitus*, recently described by J. Oppenheimer (1936*c*, p. 408). It may be noted that among the teleosts described in the literature there are some with a very variable cleavage plan, e.g., *Siphostoma Floriaæ*, *Serranus atrarius*, etc. Others have a very regular and invariable cleavage pattern, e.g. *Fundulus* and even more so *B. rerio.* The second cleavage plane appears perpendicular to the first one, the third parallel to the first, the fourth parallel to the second and the fifth parallel to the first. The sixth cleavage is the first horizontal one. In *Fundulus* the fifth cleavage is usually horizontal. The cells divide synchronously for a long time. In the film a rhythm of division can be seen still in the blastula stage. This phenomenon will be followed into details in later work.

Discussion

The most obvious point of view, from which the striking changes in the early development of the egg, in volume and shape, refraction and streaming rate may be surveyed, is that of the physical state of the cell, but it is a question how far the physical state can be determined from simple observations without experiments. It is *a priori* certain that a quantitative analysis is quite impossible in this way and that many delicate changes which occur in viscosity and surface tension do not appear as changes in shape, or in refraction, or in the rate of movement. But if we confine ourselves to the most striking visible changes, it may be possible to conclude something indirectly. The results concerning physical changes in the living egg obtained by experimental methods, microdissecting and centrifuging, are not so abundant and unquestionable that an attempt to confirm one or the other by different means would be superfluous.

Fertilization initiates a complex of processes. At first, the membrane lifts off, and an intake of water occurs. Then the egg begins to shrink and the dark granular structure of the unfertilized egg becomes even more opaque. When fixed in this stage there appear clouds of fine granules just outside the surface of the cell in the perivitelline space, and under the influence of the fixing fluid, more of the same stuff will be pressed out of the cell, as it shrinks visibly. Though faint traces of substance appear in all stages in the perivitelline space after fixation, no material is observed to come out of the cell except in the very first time after fertilization. The intake of water is at first confined to the perivitelline space; the cell itself loses some kind of albuminous substance into the hypotonic fluid, which begins to surround it. Whether a change in the colloidal state of the egg cytoplasm is involved in this process cannot be told. Very soon, however, there seems to be more evidence for an actual change. The cell begins to swell, the surface shows amœboid movement, a clear protoplasmic coat becomes visible and the content of the cell as a whole becomes more transparent. The granular structure appears coarser now. The most striking change is the flattening of the animal pole. When this occurs, the blastodisc is no longer a faintly visible concentration of granular protoplasm, but consists in a distinct clear disc. The whole animal pole, including both the yolk part and the cytoplasmic blastodisc, is flat and the yolk keeps the same flattening at the base of the blastodisc throughout the separation process and the first two cleavages, though the flat surface of the blastodisc disappears quickly. Later the yolk becomes a spherical body until the blastula stage when it again flattens, at the base of the blastodisc.

The next change is due to the hydration of the cell itself, which swells and becomes more transparent. This hydration seems to be analogous to the hydration observed in most eggs after fertilization. At this time, the last polar body is extruded, and the fused pronuclei probably come to a brief pause, as has been observed in other teleosts. The egg shows a complete resting stage before the first cleavage and the separation of protoplasm from yolk begins.

There are apparently three stages to the bipolar differentiation in the development of the zebra fish egg. At first the bipolar differentiation is indicated by the situation of the nucleus. The processes of fertilization and the following hydration bring about the concentration of a certain amount of protoplasm around the nucleus and so the blastodisc is established. A further concentration of protoplasm in the blastodisc occurs at the beginning of the first mitosis, and the process of separation comes to a rest, when the nuclei are completely divided and in a resting stage. It begins again with the prophase of the succeeding mitoses. This points to a connection between the two processes. The connection is to be sought in all probability in the physical state of the cell, which is altered during the cell division. Spek (1933) tried to determine the cause of bipolar differentiation in teleost eggs by means of his ingenious experiments using indicators on the living egg. He proved that gravitation is not a possible cause for the separation and considered surface tension likewise impossible, since the surface tension theory (Spek, 1918) requires a counterstream corresponding to the axial stream, and no such counterstream was observed. He tries to explain the bipolar differentiation chiefly by means of electrical forces, which are exerted between the different particles of the colloidal mixture. I do not have any reason to doubt this result, but in the *Brachydanio* egg it seems evident that the physical state of the cell, viscosity and surface tension, have also an important influence on the separation process. The main argument against this loses its value, because a counterstream, which has the opposite direction of the axial stream of protoplasm into the blastodisc, is actually present. In addition, there is certainly abundant indication that there is a change in viscosity and surface tension taking place at the time when the separation process begins. The initial change in the physical state starts with the hydration of the cell; the later ones begin with the cell divisions.

The changes which the cells undergo during mitosis are obvious in the living egg, though no nuclear structures are visible. The separation process is always at its climax when a cleavage plane first becomes visible in the late anaphase, it decreases rapidly while the cells round up, and the cleavage is completed, and it begins again very soon, probably with the prophase, when the surface tension of the cells seems to be lowered. The phenomenon of the "Mischzone" is not easy to explain. Whether the great differences in the volume and density of the area underneath the blastodisc are due merely to an alteration of the size of the particles in the emulsion with the changing rate of movement in the substances, or whether there is an actual change in index of refraction of the protoplasm, remains a question. In some respects the last conception seems more probable, because sections show only insignificant differences in size of the particles in the "Mischzone." Flemming (1891) found that connective tissue cells are dark in the earlier stages of mitosis and became lighter in the late anaphase. The "Mischzone" of *B. rerio* eggs appears darkest just at the anaphase.

Chambers (1920), in the course of his experimental study of the consistency and viscosity of the sea urchin egg, found that the shape of the blastomeres, round in some stages, more flattened in others, fitted well his conclusions concerning the viscosity changes as obtained with the micromanipulator. The changes in the shape of the cells of the zebra fish egg are also very striking, as has been described, and they are also immediately connected with the general physical state of the cells. Definite conclusions, however, as to the viscosity and surface tension from the shape of the cells alone would be vague without a quantitative investigation.

SUMMARY

A detailed description of the early development of the egg of *B. rerio* is presented. The observations are made chiefly on the living egg. The measurements are made on a moving picture, taken by time accelerator. This proved to be a valuable help in discovering facts which could not be detected by simple observations.

After fertilization the egg as a whole takes up water, but the cell itself shrinks. Later the cell itself begins to swell and to take up water.

The bipolar differentiation is first indicated by the position of the nucleus and a small amount of protoplasm around it. A conspicuous separation of protoplasm from yolk begins when the cell first takes up water and ceases during a complete resting phase before the first mitosis. With the beginning of the first mitosis a separation process of different nature takes place. This consists in a streaming of protoplasm through the yolk towards the blastodisc. In addition, a counterstream towards the vegetal pole is clearly visible in the film. The changes in the physical state of the cells connected with the mitoses are immediately correlated with the rhythmic separation process.

It is possible to orient the egg at the time of fertilization not only with respect to the polar axis but also with respect to the first cleavage plane.

I am deeply indebted to Professor J. W. Wilson, Brown University, for having frequently discussed the paper with me and given valuable advice regarding its form.

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THE EFFECT OF ULTRA-VIOLET LIGHT UPON CLEAVAGE IN CERTAIN MARINE EGGS

I. Some Studies on Cleavage Rate

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Introduction

Results of studies on the biological effect of radiation are reported extensively in the literature. The action of radiations of short wave length is reviewed by Packard (1931), and a splendid summary of radiation effect on living protoplasm is presented by Heilbrunn and Mazia (1936). A widespread therapeutic application of the biological action of radiation is discussed by Mayer (1925), Saidman (1925), Bach (1927), and Guillaume (1927). In the large body of experimentation upon the subject, cellular sensitivity has an important rôle in the analysis of radiation effect. Frequently this sensitivity has been regarded as susceptibility or resistance during cellular activity. It is indicated by the alteration of the reaction time of biological processes whose end-point is some well-defined morphological change which is characteristic of the normal activity of the cell. The first cell division in fertilized eggs of certain marine invertebrates is the process which has been selected as an experimental criterion by many investigators. Packard (1916) showed that cleavage rate in eggs of *Arbacia* was accelerated following exposure to radium emanations. The alteration of the rate of cell division (usually a delay in the time of cleavage) by X-rays was reported by Richards (1914) for eggs of *Planorbis;* Langendorff (1931) for eggs of *Psammechinus;* Henshaw (1932) for *Arbacia;* Henshaw, Henshaw, and Francis (1933) for eggs of *Chætopterus*, *Nereis*, and *Cumingia;* Heilbrunn and Young (1935) for *Arbacia;* and Henshaw and Francis (1936) for *Arbacia.* A similar effect by exposure of eggs to ultra-violet light is shown by Lillie and Baskervill (1922) for *Asterias* and Chase (1937) for *Urechis.*

The present work details the effect of ultra-violet radiation upon the rate of cleavage in eggs of the sand dollar, *Dendraster excentricus*, and the echiuroid worm, *Urechis caupo*. The experiments are for the purpose of determining the sensitivity of marine eggs to radiation effect when exposures are made at certain stages in development during the period from insemination to first cleavage.

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[1] Aided in part by a grant from the Committee on Radiation, National Research Council.

PROCEDURE AND RESULTS

The two forms, *Dendraster excentricus* and *Urechis caupo*, are abundant in the Monterey Bay region of the California Coast. Animals were collected and kept in aquaria supplied with well aerated, running sea water. Experiments were arranged so that eggs and sperm were obtained from freshly collected specimens. For an experiment, a quantity of gametes was used which showed high fertilization capacity (98 per cent to 100 per cent development of fertilization membranes). Eggs were placed in finger bowls containing 250 cc. of sea water and dry sperm were kept in covered Syracuse dishes. A sample from the supply of eggs was placed in sea water to a depth of

TABLE I

Stages during pre-cleavage development when eggs were irradiated

Period	Description of stages	
	D. excentricus	*U. caupo*
Before insemination	The process of maturation is completed.	The process of maturation has not begun.
15 minutes after insemination	The large sperm aster is seen and the pronuclei approach each other.	The germinal vesicle breaks down, indicating the beginning of the process of maturation.
30 minutes after insemination	Fusion of the pronuclei.	The first polar body has been extruded and the formation of the second polar body is taking place.
45 minutes after insemination	The resting nucleus stage which precedes the mitotic phases.	The second polar body has been extruded and the process of maturation is completed.
55 minutes after insemination	Phases of mitosis which lead to first cleavage.	
60 minutes after insemination		The resting nucleus stage which precedes the mitotic phases.
70 minutes after insemination		Phases of mitosis which lead to first cleavage.

2.5 mm. in each of eight thin-walled, flat-bottomed glass dishes and exposed to ultra-violet light for durations of 15, 30, 45, 60, 75, 90, 105, or 120 seconds. The eggs in each dish then were inseminated with a standard sperm suspension (a drop of dry sperm from a micropipette added to 5 cc. of sea water) and allowed to develop in a greater volume of water. A larger sample was taken from the supply of eggs, inseminated, and allowed to develop. As the eggs reached a certain stage in development (see Table I), they were placed in each of eight dishes as in the previous case and given a duration of exposure. The irradiated eggs then were allowed to continue to develop in a larger volume of water. A batch of eggs which served as the control was taken from the general supply of eggs and inseminated simultaneously with each sample of eggs which was given the treatment with the ultra-violet light. The time of insemination in controls and rayed eggs was recorded.

Caution was observed in handling eggs and their contamination was guarded against so as to insure that premature development did not occur. Experimental procedure was standardized so as to minimize the possibility of aberrant results. The gametes, from the time of their removal from the animals to the end of an experiment, were kept at a regulated temperature of approximately 20° C. Before exposures the ultra-violet lamp was allowed to burn 10 minutes and traces of ozone were removed. During exposures dishes of eggs were placed at a distance of 30 cm. from the source of the light and the eggs were exposed to the full spectrum of the mercury arc. Radiations were made with an Analytic Model Quartz Lamp, Hanovia, which operated on a 110 volt circuit—alternating current—60 cycles—five amperes. Approximations of the intensity of the output of ultra-violet radiation at the distance were made frequently during the experiments and the variation detected was negligible.

The first three cleavages of controls and irradiated eggs were observed and the time of each was recorded at the moment when the reacting half of 10 eggs had begun to divide. Ten recordings were made for each sample of eggs, both rayed and controls, in order to obtain the time of the cleavage of the reacting half of 100 eggs for each. These observations were repeated nine times during the course of an experiment. Twelve experiments were run for each species so that the data are based upon the reacting half of 12,000 eggs at each exposure. Since the experimental criterion was the time of 50 per cent cleavage no data were included for eggs which showed a lower percentage. In all cases measurements on irradiated eggs were rejected when the controls failed to show 98 to 100 per cent first cleavage.

Measurements of the actual time from insemination to a particular cleavage are given for eggs of *Dendraster* and *Urechis* respectively, in Tables II and III. It is revealed clearly that the time-lapse from insemination to first cleavage in eggs of both species increases with increase in length of exposure, followed by a similar effect on the rate of the subsequent two-cell divisions. Delay in segmentation time occurs regardless of the stage in development when eggs were rayed, being greater at longer exposures for certain stages than for others. The exception is to be found when eggs of the sand dollar were rayed just before first cleavage.

Table II

Average time-lapse (minutes) from insemination to the first three cleavages in eggs of *Dendraster excentricus* exposed to ultra-violet light for varying durations at a distance of 30 cm. Each value is the average time-lapse from insemination to the particular cleavage for the reacting half of 12,000 eggs. In the controls the average time-lapse from insemination was 58.2 minutes for first cleavage, 87.8 minutes for second cleavage, and 117.4 minutes for third cleavage.

Duration of exposure (seconds)	Periods when eggs were exposed and time (minutes) from insemination to each of the first three cleavages														
	Before insemination			15 minutes after insemination			30 minutes after insemination			45 minutes after insemination			55 minutes after insemination		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
15	84.3	135.4	178.4	78.7	115.8	166.7	59.3	88.3	133.8	57.4	101.2	127.4	56.8	109.1	180.8
30	85.9	138.3	193.4	94.9	137.2	204.8	61.2	91.9	151.6	57.9	110.9	139.2	55.6	117.9	191.8
45	88.1	168.3	——	95.4	143.5	208.1	62.5	114.1	176.5	59.0	150.5	187.8	57.8	120.8	217.5
60	90.9	174.5	——	99.4	141.8	227.6	65.0	124.7	171.7	59.4	164.8	207.3	56.3	133.5	223.6
75	94.8	——	——	101.6	148.9	——	69.5	117.2	169.8	60.6	173.2	——	57.3	147.4	237.2
90	97.0	——	——	104.8	155.7	——	73.2	121.5	163.4	62.7	177.3	——	56.9	169.7	——
105	——	——	——	108.7	173.4	——	78.8	114.8	168.2	68.7	——	——	58.1	168.3	——
120	——	——	——	111.9	169.7	——	83.1	117.3	174.1	82.3	——	——	58.9	174.8	——

The time-lapse measurements for irradiated eggs were compared with similar measurements for controls and the relation between the measurements is presented as the per cent increase in time-lapse (delay in cleavage time). Graphs of the percentage increase in the time-lapse for cleavage in rayed eggs which reveal the differences in the extent of radiation effect during the various stages in development are shown in Figs. 1 and 2.

In the text figures abscissæ show durations of exposure and ordinates indicate cell division rate as the percentage increase in time-lapse from insemination to cleavage. The groups of curves, Fig. 1*A* and

TABLE III

Average time-lapse (minutes) from insemination to the first three cleavages in eggs of *Urechis caupo* exposed to ultra-violet light for varying durations at a distance of 30 cm. Each value is the average time-lapse from insemination to the particular cleavage for the reacting half of 12,000 eggs. In the controls the average time-lapse from insemination was 70.3 minutes for first cleavage, 101.8 minutes for second cleavage, and 132.5 minutes for third cleavage.

Duration of exposure (seconds)	Periods when eggs were exposed and time (minutes) from insemination to each of the first three cleavages																	
	Before insemination			15 minutes after insemination			30 minutes after insemination			45 minutes after insemination			60 minutes after insemination			70 minutes after insemination		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
15	70.7	107.3	197.4	71.2	109.5	146.3	73.3	107.3	138.3	71.8	105.6	134.6	69.6	102.5	136.7	69.9	98.9	123.6
30	72.8	128.5	238.1	74.8	123.7	197.6	75.4	125.6	168.1	79.2	108.6	148.2	71.0	109.3	148.3	71.1	101.3	132.8
45	—	—	—	77.4	—	—	84.7	—	—	89.7	150.3	—	72.4	113.5	157.2	71.4	109.6	146.4
60	—	—	—	78.7	—	—	98.5	—	—	100.9	—	—	75.6	117.1	169.2	76.8	115.5	158.5
75	—	—	—	—	—	—	—	—	—	—	—	—	82.5	150.3	—	83.2	128.7	172.8
90	—	—	—	—	—	—	—	—	—	—	—	—	91.9	—	—	92.7	—	—

Fig. 2*A*, show the sensitivity of the eggs to ultra-violet radiation when exposures were made at certain stages in development before initial segmentation. The groups of curves, Fig. 1, *B* and *C*, and Fig. 2, *B* and *C*, show the extent to which radiation treatment of the eggs before first cleavage affects the rate of subsequent cell divisions.

Discussion

The interpretation of results on the effects of radiant energy upon living cells is based upon factors which are chiefly of a biological or a physico-chemical nature. The significance of the biological aspect of the problem is emphasized by Failla (1924). A factor of importance from this point of view is the fundamental difference in cellular activity, especially in animal ova, during early stages in development.

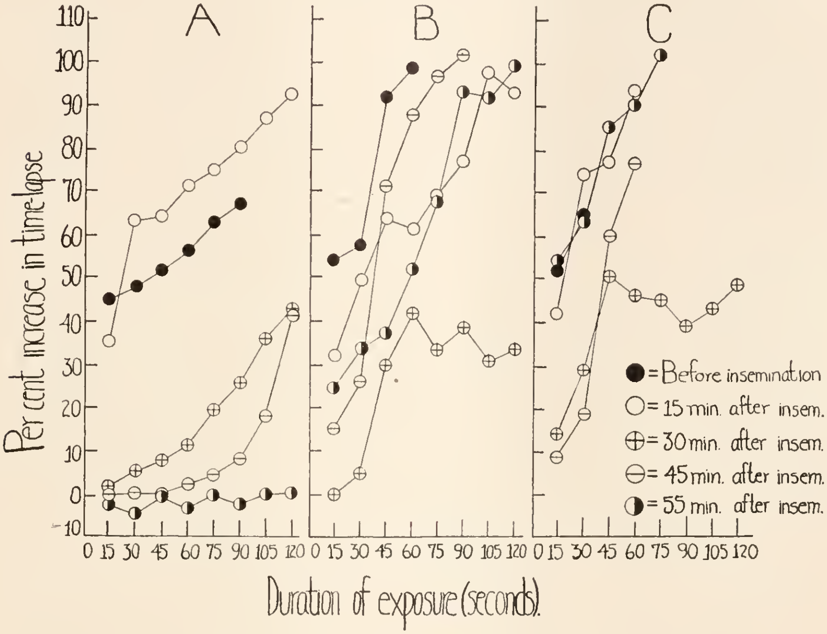

Fig. 1. Curves showing the rate of cleavage in eggs of *Dendraster excentricus* exposed to ultra-violet light at different stages in development. First, second, and third cleavage (*A*, *B*, and *C*).

Since frequent use is made of the rate of first cleavage in dividing eggs as an indicator of radiation effect, it is essential to know the relation between response to exposure and the characteristics of early (pre-cleavage) development. A greater variation occurs in the sequence of events which leads to the initial segmentation of the fertilized egg than is found during the period which precedes any subsequent cleavage in the same egg or during inter-division phases of other dividing cells. Eggs fall in categories with respect to the biological processes which follow insemination and lead to first cleavage, Lillie and Just (1924) and Wilson (1925). The eggs of *Dendraster* *excentricus* and *Urechis caupo* are so widely separated with regard to internal phenomena during the period of pre-cleavage development that a consideration of the results which have been presented is of interest because of its bearing upon the study of cellular sensitivity to radiation. It is probable that variations in developmental processes,

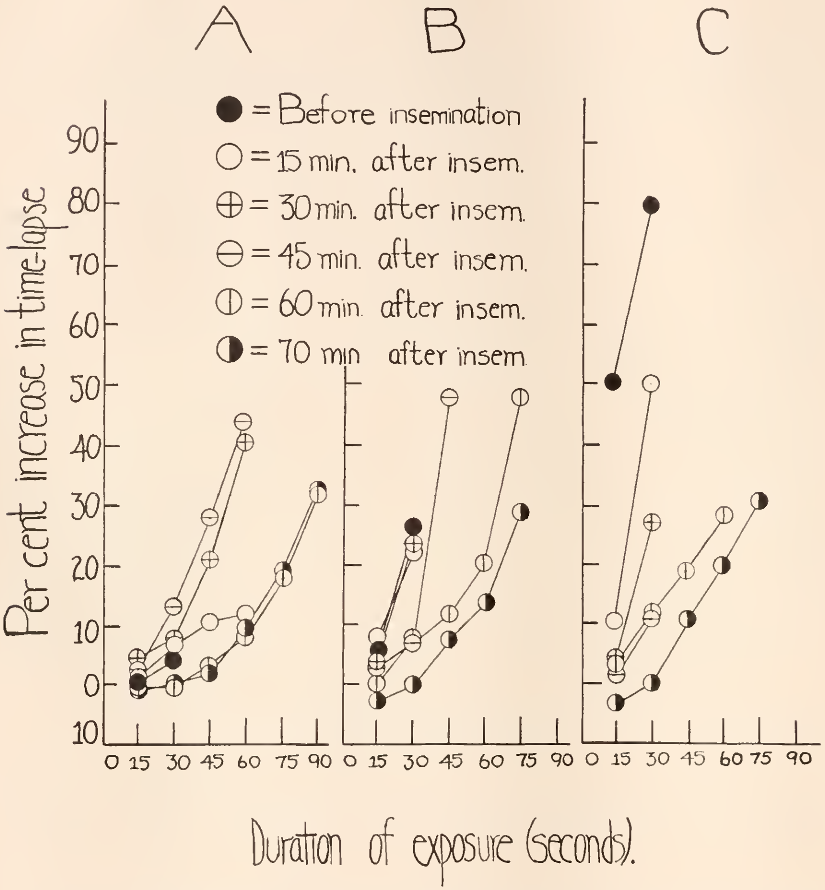

FIG. 2. Curves showing the rate of cleavage in eggs of *Urechis caupo* exposed to ultra-violet light at different stages in development. First, second, and third cleavage (*A*, *B*, and *C*).

and the underlying complex of reactions, may be factors in the analysis of radiation effect when the rate of first cleavage in egg cells is the criterion of experimentation.

Comparison of the data in Tables II and III, and of the groups of curves in Fig. 1 with the curves in Fig. 2. reveals a marked difference in the response of egg cells to irradiation during the stages of pre-cleavage development which precede the first cell division. Susceptibility of the eggs to radiation effect (maximum delay in cleavage time) occurs during the period from insemination to the resting nuclear stage. During the mitotic phases which lead to initial segmentation, eggs of the two species are resistant (minimum delay in cleavage time) to the effect of ultra-violet rays. Since results from X-ray studies show a greater effect of exposure during certain mitotic phases,—Richards (1914) for *Planorbis*, found X-rays most effective during the metaphase,—a matter of interest is the fact that no marked susceptibility to ultra-violet radiation was exhibited by eggs of *Dendraster* and *Urechis* during the various phases of the mitotic cycle.

The results cited give evidence of a differential response of fertilized eggs to irradiation which may be correlated with processes involved in pre-cleavage development. Suppression of maturation in certain eggs following exposure to ultra-violet light, Just (1933) for eggs of *Nereis limbata* and Chase (1937) for eggs of *Urechis caupo*, may be a factor in the delay of cleavage in irradiated eggs. Some other factor exists for eggs of *Dendraster*, since the maturation process is not a part of pre-cleavage development. A reasonable probability is the metabolic process which parallels cellular activity during pre-cleavage development. Whitaker (1931) reported a decline in the rate of oxygen consumption during the first 40 minutes after fertilization for eggs of *Nereis limbata* while eggs of sea urchins show an increased rate of metabolism during the first hour after fertilization (see comparisons by Whitaker, 1933). In view of the similarity of earliest development in eggs of *Nereis* and *Urechis* and in eggs of sea urchins and *Dendraster*, it is possible that approximately equivalent rates of metabolism are concomitant with developmental processes which are suppressed or inhibited by ultra-violet radiation. Alteration of metabolic rate conceivably may cause acceleration or retardation of phases of cellular activity which underlie cell division, and thus affect segmentation tempo.

Another agency which may affect the rate of cell division in developing eggs is the change in the consistency of egg protoplasm following exposure to radiation. During the experiments eggs of both species were overexposed (rayed for durations of four to seven minutes) at different stages in development. When the eggs were observed about 20 minutes after irradiation a very definite flowing of the endoplasm was seen. Streaming of the inner protoplasm of the eggs was not observed for any of the durations of exposure which are reported in the data, but it is possible that an unobservable change in viscosity may have followed some of the longer irradiations. Measurements of the changes in the viscosity of *Arbacia* egg protoplasm following exposure to ultra-violet light are presented by Heilbrunn and Young (1930). According to the changes in the viscosity of egg protoplasm it is reasonable to assume that reactions may be affected which influence the rate of cleavage.

The delay in segmentation time of the second and the third cleavages (see Fig. 1, *B* and *C*, and Fig. 2, *B* and *C*) occurs as a result of the irradiation of the egg during periods of development before first cleavage. Two explanations of the effect are: (*a*) initial injury of the egg caused by the pre-cleavage exposures; (*b*) a latent effect of radiation which varies in extent for each of the two cell divisions with respect to the stage in development when exposures are made. In both cases failure of recovery from the effect of radiation is indicated, and the effect upon the rate of cleavage is apparently a function of the duration of exposure. The theory that the eggs are injured in some way is the more tenable, although there is a strong evidence of a latent effect of radiation, especially in eggs of *Dendraster excentricus*, which were rayed just before the first cleavage.

There is need of further studies on the effect of radiation upon developing eggs which, from a purely biological point of view, may serve for more adequate analysis of radiation effects upon living cells. Work is now in progress on the percentage of cleavage in developing eggs as an indicator of susceptibility or resistance to irradiation.

SUMMARY AND CONCLUSIONS

1. The effect of ultra-violet light upon the rate of the first three cell divisions in eggs of *Dendraster excentricus* and *Urechis caupo* is an increase in segmentation tempo which is apparently a function of duration of exposure. This delay in cleavage time occurs regardless of the period in development from insemination to first cleavage when exposures are made.

2. The sensitivity of fertilized eggs to radiation effect is dependent upon the stage in pre-cleavage development when exposures are made. Since developmental processes vary for eggs of different species, during the period from insemination to first cleavage, differences in the degree of sensitivity may be expected.

3. Evidence for a differential response of fertilized eggs to irradiation is presented which may be correlated with radiation effect upon a process of pre-cleavage development, or upon underlying reactions which are concomitant with phases of cellular activity which lead to cell division.

4. The viability of fertilized eggs of the two species varies over the range of exposures, longer durations preventing cleavage in eggs of *Urechis*. In view of this fact, adequate measurements of segmentation rate may not be recorded at all exposures to ultra-violet light.

5. The analysis of radiation effect upon living cells, from a purely biological point of view, should be based upon conclusive data which take into consideration the vast differences in cellular activity during pre-cleavage and inter-division stages. In this connection, it is suggested that a more appropriate criterion for cellular sensitivity to radiation effect may be the determination of the percentage of cleavage (survival) in egg cells of the sand dollar-sea urchin type of developmental activity.

The writer wishes to express his gratitude to Dr. W. K. Fisher and the staff of Hopkins Marine Station for their many courtesies and assistance. He is also indebted to Professor Frank Coleman, Department of Physics, Howard University for technical advice and assistance.

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THE MORPHOLOGY AND LIFE CYCLE OF THE TREMATODE HIMASTHLA QUISSETENSIS (MILLER AND NORTHUP, 1926)

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The trematodes of the family Echinostomatidæ comprise a large number of genera which infest the alimentary tract of birds and mammals. The family and several genera were characterized by Dietz (1910). In a preliminary paper, Dietz (1909) had erected the genus *Himasthla*, with *H. rhigedana* as the type species, and in it he included *H. alincia* Dietz, *H. leptosoma* (Creplin), *H. militaris* (Rudolphi), *H. elongata* (Mehlis), and *H. secunda* (Nicoll). Linton (1928) described specimens which he identified as *H. elongata* (Mehlis), and others which he named *H. incisa* new species. A new human parasite, *H. muehlensi*, was described by Vogel (1933), who included a tabular description of all known species of *Himasthla*. Palombi (1934) described the metacercaria of a new species, *H. ambigua*, from the gills of *Tapes decussatus*.

The first complete life history of an echinostome to be recorded was that of *Echinostoma revolutum* by Johnson (1920). Subsequent life cycles, demonstrated experimentally, include that of *Hypoderæum conoideum* (Bloch) by Mathias (1925), of *Echinoparyphium recurvatum* (von Linstow) by Mathias (1927), of *Echinoparyphium aconiatum* Dietz by Riech (1927), of *Euparyphium murinum* Tubangui by Tubangui (1932), of *Euparyphium ilocanum* (Garrison) by Tubangui and Pasco (1933), of *Euparyphium malayanum* by Rao (1933), of *Echinoparyphium recurvatum* by Rašín (1933), of *Nephrostomum ramosum* by Azim (1934), of *Echinostoma coalitum* by Krull (1935), and of *Echinostoma revolutum* by Beaver (1937). The monograph of Beaver contains a review and analysis of the earlier papers. He noted certain discrepancies in the account of Johnson (1920) and suggested that the observations were made on material of more than one species. Indeed, the older descriptions are so incomplete and so inaccurate that it is exceedingly difficult to determine how many times one species has been redescribed or how many species have been confused. This condition is particularly prominent and perplexing when both adult and larval stages are considered together. Referring to descriptions of a group of eleven species of echinostome cercariæ, Beaver, p. 17, stated, "While it is not certain that they all are identical, it is certain that they can not be distinguished from each other." He pointed out that *Echinostoma revolutum* is cosmopolitan in geographic distribution and has little specificity in any of its parasitic stages. Using biometric methods on specimens reared under known, controlled conditions, he measured the normal variation in this species, noting especially the modifications induced in genetically similar material as a result of development in different avian and mammalian hosts. In the light of the more complete and precise knowledge concerning *E. revolutum*, Beaver made a critical examination of the descriptions of related species. Eight of them were reduced to synonymy and eight others were listed as of doubtful standing.

Other important contributions to the life history of these parasites include those of Ciurea (1920) and the reports of several Japanese investigators whose accounts, published in Japanese, are unfortunately not readily available. In the life cycles described, the cercariæ have been found to encyst in various mollusks, fishes and tadpoles.

Although the life history has not been completely demonstrated previously for any other species of *Himasthla*, there have been certain important and valuable contributions. Villot (1879) redescribed *H. leptosoma* (Creplin) and identified an encysted metacercaria from *Scrobicularia tenuis* as a stage in the life cycle of the worm. He traced the successive stages of development from the encysted metacercaria to the mature adult in the intestine of *Tringa variabilis*. Nicoll (1906*a*, 1906*b*) showed the close morphological agreement between an echinostome metacercaria, which he found encysted in *Cardium edule*, *Mytilus edulis* and *Mactra stultorum*, and an adult which he described as *Echinostomum secundum*. Lebour (1908) confirmed the account of Nicoll and reported an echinostome cercaria with twenty-nine cephalic spines which she believed to be the larva of *E. secundum*. Experimental infection under controlled conditions was not secured.

The life cycle of an echinostome cercaria which occurs in *Nassa obsoleta* at Woods Hole, Massachusetts, has been experimentally traced and a preliminary note was published, Stunkard (1934*a*). A more extended account was presented (Stunkard, 1937). There is adequate evidence that the cercaria is identical with the one described by Miller and Northup (1926) as *Cercaria quissetensis* and the adult is specifically identical with certain of the worms identified by Linton (1928) as *Himasthla elongata*. Since the sexually mature specimens are specifically distinct from *H. elongata*, the new combination *Himasthla quissetensis* was adopted.

To be convincing, life history studies must be carried on under well controlled conditions. The hosts used for experimental infections must be free from a previous infection which could be confused with the experimental one, or the course of development of the parasite must be followed at such short intervals that the organism can be identified at every stage. In the present study, the gulls and terns used as final hosts were removed from the nesting grounds as soon as they were hatched and, since they had never been fed by their parents, were known to be free from infection. They were fed on fish which contained no trematode larvæ, so the experimental infection was not complicated. Rats used in the experiments were laboratory raised and harbored no trematode parasites. The cercariæ of *H. quissetensis* were observed to penetrate into the gills and other organs of mollusks and encyst there. These encysted metacercariæ were fed to rats, terns and gulls, all of which were known to be free of trematode infection. The metacercariæ are infective for the final host soon after encystment and undergo little development in their cysts. It appears, therefore, that the cyst serves as a protective device to carry the larva through the acid digestion of the stomach, and that the metacercaria emerges in the small intestine of the bird or mammal. Successive stages of development were recovered from the intestine of experimentally infected birds.

The Sexual Stage in the Life Cycle

The Adult

About two hundred worms (Fig. 8) were recovered from the intestine of a herring gull, *Larus argentatus*, thirty-one days after the ingestion of experimentally infected mollusks. The shape of the body is portrayed in the figure. It is capable of much elongation and contraction and the appearance of the worms and their internal organs is modified accordingly. All of the specimens contain eggs and although the terminal portion of the uterus is usually empty, many eggs were being passed in the feces of the bird. It is possible that the region of the body immediately behind the cirrus sac would have been slightly wider if the worms had been older or more completely matured. When alive and greatly extended the sides are smooth but in all well-fixed specimens the contraction of circular and longitudinal muscles gives the edges of the body a crenated or ringed appearance. Fixed and stained specimens measure from 5 to 10.2 mm. in length and 0.5 to 0.75 mm. in greatest width. The anterior end bears a reniform collar, interrupted ventrally, and at this level the body measures about 0.3 mm. in diameter. On the collar there are thirty-one cephalic spines, arranged in a single row except for the lateral corners where two spines are situated between and behind the others. The spines measure from 0.045 to 0.058 mm. in length and 0.014 to 0.020 mm. in width. The corner spines are only slightly shorter than those in the row. The size and arrangement of the cephalic spines agree with the description and figure of these structures on the worms identified as *H. elongata* by Linton (1928). In the preacetabular region the cuticula is beset with flattened, scalelike spines which are largest near the anterior end and become progressively smaller posteriorly. The acetabulum measures 0.2 to 0.4 mm. in diameter and it is situated about the same distance behind the oral sucker. In a specimen 7.8 mm. long, the acetabulum is 0.36 mm. in diameter.

Digestive System.—The oral sucker is subterminal, spherical to ovoid in shape, and measures from 0.07 to 0.125 mm. in length by 0.1 to 0.135 mm. in width. The prepharynx is short and the pharynx measures from 0.1 to 0.13 mm. in length by 0.06 to 0.09 mm. in width. The esophagus extends to the level of the anterior margin of the acetabulum and the ceca to the posterior region of the body.

Male Genital System.—The testes are elongate, faintly lobed structures, situated in the caudal third of the body. The anterior testis measures 0.5 to 1 mm. in length and 0.25 to 0.33 mm. in width. The posterior testis is usually somewhat longer and measures from 0.7 to 1.1 mm. in length and 0.2 to 0.26 mm. in width. The testes are close together in small or contracted specimens but in larger or extended ones they are separated by a distinct interval. The sperm ducts are very small and could not be observed in whole mounts. They were traced in one series of sections. A vas deferens arises from the anterior, ventral surface of each testis and passes forward, median and ventral to the cecum, the duct from the anterior testis on the left and that from the posterior testis on the right side of the body. Both open into the caudal end of the cirrus sac where they discharge into a large, coiled, seminal vesicle which occupies the posterior third to half of the cirrus sac. The cirrus sac is long and the postacetabular portion winds about on the dorsal side of the body. Its extent is indicated in the figure but measurements of length in an organ of this shape are not significant. The cirrus is armed with small recurved spines. The genital pore is median, at the anterior border of the acetabulum.

Female Genital System.—The ovary is spherical to oval, usually broader than long, 0.1 to 0.22 mm. in diameter. It is situated near the median line, a short distance in front of the cephalic testis. The oviduct arises at the caudal end and passes backward to the oötype which is surrounded by the cells of Mehlis' gland. From the oötype, Laurer's canal proceeds in a sinuous course to the dorsal surface. The vitellaria are lateral to the intestinal ceca and extend from a level slightly in front of the caudal end of the cirrus sac to the posterior end of the body. In the fields on either side of the testes, ordinarily there are no vitelline follicles and a duct connects the separated portions of the glands. In one specimen a few small follicles were present on one side of the posterior testis. There is a cluster of follicles on either side at the level between the testes. Immediately in front of the cephalic testis, ducts arise from the ventral sides of the vitellaria and increase in size as they pass dorsally and medially where they unite to form a short common duct which discharges into the oötype. There is no seminal receptacle, but the initial portion of the uterus is filled with spermatozoa. The uterus extends in a sinuous course, backward to the level of the cephalic testis and then forward to the metraterm which lies below the caudal end of the cirrus sac. Both metraterm and cirrus sac pass on the dorsal side of the acetabulum. The eggs are large, oval, operculate, thin-shelled, and measure from 0.1 to 0.125 mm. in length by 0.06 to 0.08 mm. in width. They do not develop in the worm and those in the terminal part of the uterus contained only a fertilized ovum and masses of vitelline cells. Indeed, the terminal half of the uterus contains very few eggs, and it appears that they are passed rapidly through this portion of the organ.

The Asexual Stages

The cercariæ develop in rediæ which occupy the interlobular lymph spaces in the digestive gland of *Nassa obsoleta*. Experimental infection of the snail was not secured as the work was done during the summer at the Marine Biological Laboratory, Woods Hole, Massachusetts, and development of the eggs was so slow that hatching was not obtained before the end of the season. Johnson (1920) and Beaver (1937) reported that eggs of *Echinostoma revolutum* hatch in three to four weeks after they are passed in the feces. In *Euparyphium ilocanum*, according to Tubangui and Pasco (1933), the eggs require from two to five or six weeks to complete their development. Rašín (1933) found that eggs of *Echinoparyphium recurvatum* require two weeks at 25° and three weeks at 20° for the development of the miracidium. In all echinostome species in which development has been studied, the eggs contain unsegmented ova when they are passed. From two to five weeks, depending on the temperature, are required for development of the miracidium which then emerges from the shell and penetrates into the snail which serves as the first intermediate host. The development of all members of the family Echinostomatidæ undoubtedly follows the same general plan and accordingly it is probable that the miracidia of *H. quissetensis* complete their development in the eggs, emerge and penetrate into *Nassa obsoleta*. For *E. revolutum*, Johnson (1920) postulated that the mother rediæ were derived by metamorphosis from miracidia. Later studies on other echinostomes, however, indicate that in this family as in other digenetic trematodes, the miracidium metamorphoses into a sporocyst and the next generation is produced in the sporocyst. It is, of course, possible that there may be only a single redia in the sporocyst as reported by Linton (1914) for *Parorchis avitus* and by Stunkard (1934*b*) for *Typhlocœlum cymbium*. In the latter cases, however, the mother redia is well developed in the miracidium.

In natural infections of *Nassa obsoleta*, the interlobular lymph spaces in the digestive gland are filled with developing rediæ and cercariæ. Often the gland is much atrophied and the parasites are present in enormous numbers. In a heavy infection there may be thousands of large rediæ, filled with developing cercariæ. In addition there may be hundreds of small rediæ, often not more than one-half the size of a mature cercaria, free in the tissues of the snail. Their number is too large to believe that they have been formed in a primary sporocyst and, since they are of different sizes, they must have been produced more or less continuously. Thousands of rediæ have been taken from crushed snails and dissected under a binocular microscope but none was ever found in which young rediæ could be positively identified. It is impossible to determine whether the germinal masses in a redia are developing rediæ or cercariæ, and in all cases where development had proceeded to such a stage that positive identification could be made, the larvæ were cercariæ. The relative numbers of rediæ of different sizes indicated strongly the existence of two generations of rediæ but dissections gave no certain evidence of mother rediæ.

In order to discover the source of the small rediæ, a well-infected snail was cut in serial sections. Large numbers of small rediæ were found in the tissues and others were found (Fig. 1) within the body of mother rediæ. The identification is unmistakable. The pharynx of a redia can be distinguished from the oral sucker of a cercaria by the number and arrangement of the nuclei. The feet of the daughter redia are clearly visible, and there is no oral sucker or acetabulum. An intestine is present, although empty and collapsed. Behind the intestine there is a cleft which contains germinal cells and small germ balls, young stages of the next or cercarial generation. In addition to a daughter redia, each mother redia contained from six to sixteen other germinal masses but they were undifferentiated and their appearance gave no indication concerning their nature. It would be impossible to determine whether they were young rediæ or cercariæ if it were not for the presence of the daughter redia in the same mother redia. This discovery explains the difficulty concerning the source of the small rediæ in the tissues. The daughter rediæ are produced singly and emerge from their parental generation at a relatively early stage, before they can be distinguished readily and with certainty from developing cercariæ. There is no evidence to suggest that both rediæ and cercariæ are produced in the same redia.

The Redial Generations

In early stages mother and daughter rediæ do not differ essentially from each other in size, shape, or general appearance. The two generations can be distinguished only by their progeny and by the changes which the development of the filial generation produces in them. The mother rediæ produce daughter rediæ which mature singly, whereas the daughter rediæ produce cercariæ which mature in numbers, and when filled with cercariæ the daughter redia is much distended. Mature mother rediæ (Fig. 1) in the tissue of the snail measure from 0.5 to 0.7 mm. in length and 0.14 to 0.2 mm. in width. The pharynx measures 0.05 to 0.06 mm. in diameter.

The smallest daughter redia which was sufficiently developed to be recognized is the one found in a mother redia (Fig. 1). It measures 0.13 mm. in length and 0.056 mm. in width. The pharynx is 0.033 mm. in diameter. There are both anterior and posterior locomotor appendages or "feet." Older daughter rediæ (Fig. 2), which have emerged from their parental generations and are actively migrating in the tissues of the snail, measure from 0.2 to 0.3 mm. in length and 0.1 to 0.14 mm. in width. At this stage the pharynx measures approximately 0.04 mm. in diameter, the intestine is filled with food material and extends to the level of the posterior locomotor appendages. Around the posterior part of the intestine there is a body cavity which contains germinal cells. As the rediæ mature, the body wall becomes filled with orange pigment, the intestine and anterior locomotor appendages remain small, while the caudal region of the body and posterior locomotor appendages increase in size. The body cavity enlarges, becomes filled with cercariæ in various stages of development (Fig. 3), and the birth pore becomes functional. The redia is motile and the body may assume different shapes. It may contract to a short, cylindrical form in which the posterior locomotor appendages are hardly visible, or it may extend to a long vermiform shape in which the caudal end and posterior locomotor appendages form a tripodal support for the forward protrusion of the body. The anterior end is especially mobile. In front of the pharynx there is an active oral funnel or collar. It may form a small protuberance, about one-half as wide as the rest of the body, which disappears on retraction. Mature daughter rediæ (Fig. 3) measure from 0.8 to 1.6 mm. in length and from 0.1 to 0.25 mm. in width. The pharynx is 0.05 to 0.065 mm. in diameter.

The Cercaria

During the past seven seasons over ten thousand specimens of *Nassa obsoleta* have been examined for larval trematodes. They were isolated for forty-eight hours to find the specimens from which cercariæ were emerging. About 1 per cent of the snails was infected with *Cercaria quissetensis* although cercariæ emerged spontaneously from only about one-half of these specimens. The other infections were discovered on crushing and examining the snails. Four snails were infected with both *C. quissetensis* and *C. lintoni.*

After emergence from the snail the cercaria of *Himasthla quissetensis* swims actively for six to twelve hours. In swimming, the body is bent ventrally until it is almost spherical; the tail is extended and lashes vigorously. The swimming movement causes the cercariæ to rise in the water and during the period of active swimming they are uniformly distributed throughout a large container. Occasionally, for a few seconds the tail may cease to beat while the larva elongates and manifests a serpentine movement. When the tail is quiet, the larva slowly sinks. After six to ten hours, the larvæ do not rise high in the water

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Abbreviations

ac	acetabulum
al	anterior locomotor appendage
cd	excretory collecting duct
cs	cirrus sac
dr	daughter redia
gm	germinal mass
in	intestine
os	oral sucker
ov	ovary
ph	pharynx
pl	posterior locomotor appendage
ts	testis
ut	uterus
vt	vitellaria

FIG. 1. Section of mother redia, showing daughter redia and germinal masses The daughter redia is 0.13 mm. long.

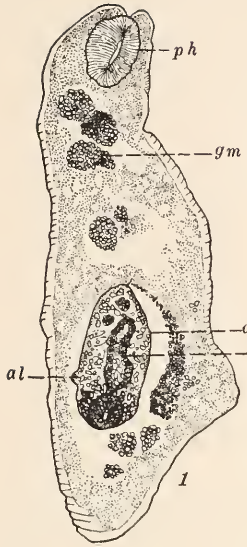
FIG. 2. Daughter redia after emergence from mother redia; drawn from a living specimen. Daughter redia is 0.2 mm. long.

FIG. 3. Mature daughter redia filled with cercariæ; drawn from a living specimen

FIG. 4. Cercaria; drawn from a fixed and stained specimen.

FIG. 5. Metacercaria; drawn from a living specimen.

PLATE I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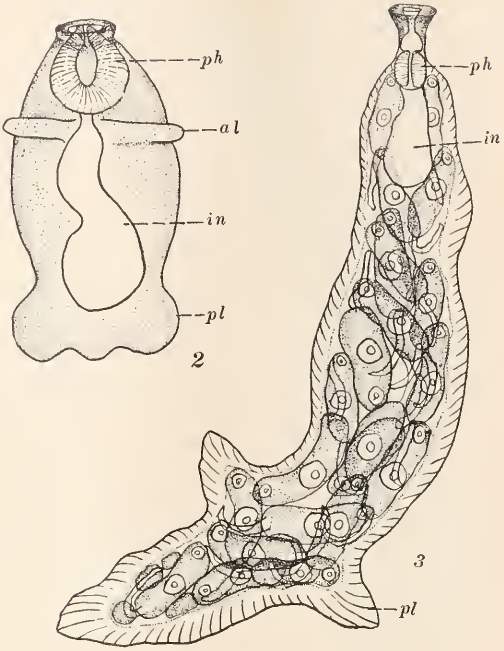
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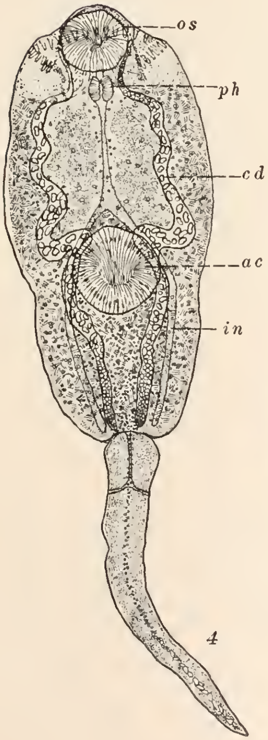
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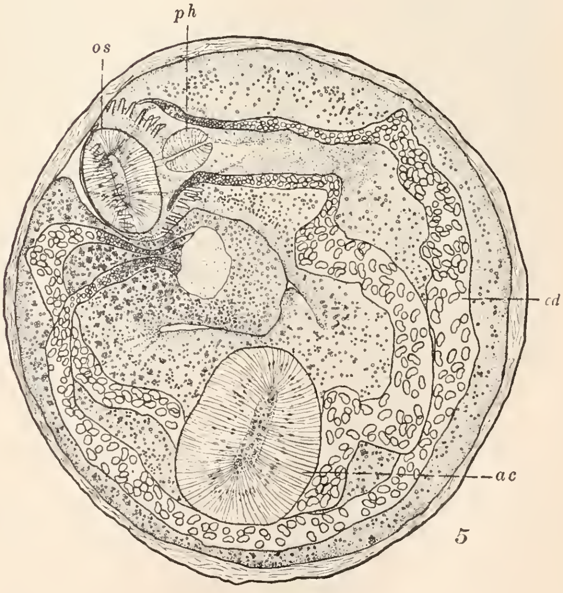

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and either swim near the bottom or creep by use of the suckers. When creeping, the tail is quiet. A report on the behavior of the cercariæ in normal and dilute sea water was made by Stunkard and Shaw (1931). After twenty-four hours in sea water there is a tendency for the cercariæ to encyst. If they do not encyst, they die in about thirty-six hours.

Fully developed, naturally emerged cercariæ (Fig. 4) are elongate oval in outline. When extended the body is very narrow and when contracted the anterior end is much widened. The body measures from 0.28 to 0.65 mm. in length and 0.06 to 0.15 mm. in width. When the larva is extended, the oral sucker is protruded, and when retracted, the anterior end of the body is flattened. On the anterior portion of the body and on the tail there are small papillæ, each of which bears a delicate, bristle-like process. The base of the tail is slightly subterminal and may be either wide or narrower than the posterior tip of the body. Contracted, the tail measures about 0.17 mm. in length and 0.048 mm. in width at the base. It is almost round in cross-section and gradually tapers to the tip. In swimming, the tail extends two to three times its length when contracted and lashes so violently that when the body is attached firmly, it may tear itself loose. The anterior end of the body is thickened to form a collar which bears the heavy spines characteristic of the species. The spines can be seen only under favorable optical conditions and it is very difficult to count them. The acetabulum is situated behind the middle of the body and measures from 0.07 to 0.085 mm. in diameter. Under slight pressure it may be as large as 0.084 to 0.091 mm. in diameter.

The digestive system may be demonstrated clearly by the use of neutral red. The oral sucker is 0.04 to 0.045 mm. in diameter. The pharynx is spherical to oval, about 0.018 mm. in diameter, situated approximately the same distance behind the oral sucker. The esophagus extends almost to the level of the acetabulum and the ceca terminate near the posterior end of the body.

Unicellular glands are abundant throughout the body. In the region between the suckers there are many penetration glands with granular cytoplasm. They are lobed and their number could not be

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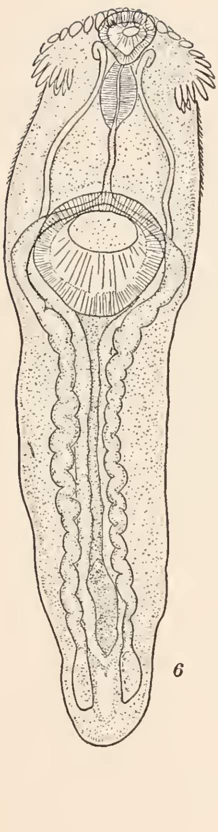
Fig. 6. Immature worm, 3 days in tern; drawn from fixed and stained specimen.

Fig. 7. Immature worm, 12 days in gull; drawn from fixed and stained specimen.

Fig. 8. Adult worm from the intestine of *Larus argentatus*, thirty-one days after cyst was fed; drawn from a fixed and stained specimen.

Plate II



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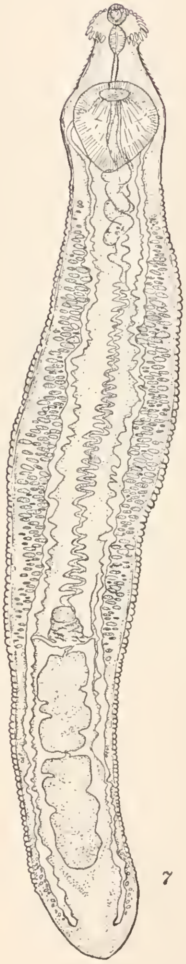

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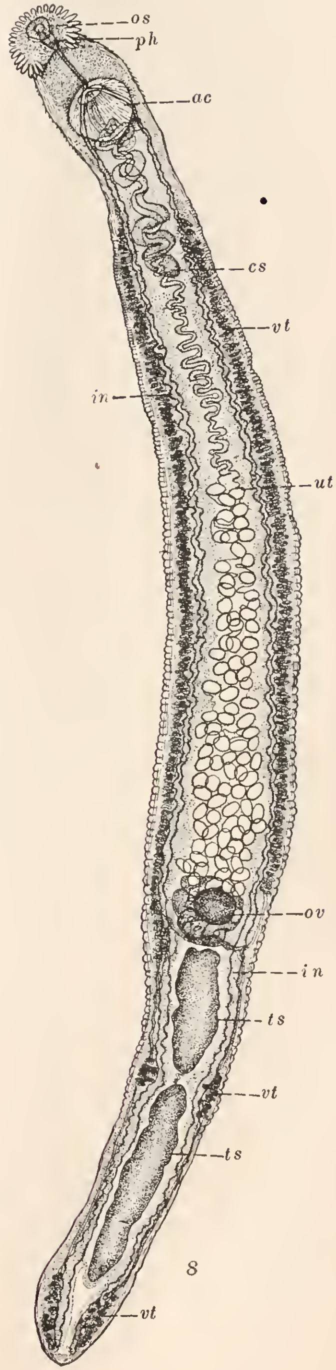
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determined. They do not stain differentially with neutral red. Ducts pass forward on the dorsal side of the body to open at the anterior tip. Much of the body surface on the dorsal side is underlaid with cystogenous glands, in which the secretion occurs in the form of bacilliform rods. Ventrally there are glands which stain intensely with eosin and erythrosin.

The genital anlage consists of a cluster of deeply-staining cells near the caudal end of the body and a row of cells which extends forward to a second cluster near the anterior margin of the acetabulum. The posterior group is the rudiment of the gonads and the anterior one of the copulatory organs.

The excretory system is difficult to trace, because in mature cercariæ the body is so filled with glandular cells, and in immature ones the tissues are so fragile that they will not stand the amount of pressure necessary to demonstrate the tubules and flame cells. There are two excretory pores, one on either side, near the proximal end of the tail. The ducts from the pores unite in the base of the tail and open into a small vesicular bladder in the caudal end of the body. From the bladder, collecting ducts pass forward on each side. These ducts are median to the ceca. They bend mediad in front of the acetabulum and then cross, ventral to the intestinal ceca, to the lateral sides of the body where they continue anteriad to the level of the oral sucker. The collecting ducts contain concretions; those near the ends of the ducts are small, 0.001 to 0.002 mm. in diameter, while those in the region between the suckers are large and measure about 0.005 by 0.009 mm. Near the anterior end of the body the collecting ducts become continuous with the recurrent excretory tubules which pass backward almost to the posterior end of the body. At their caudal ends the recurrent tubules divide to form the secondary and tertiary tubules which lead to the flame cells. The exact arrangement of these tubules and number of flame cells were not determined.

The Metacercaria

Occasionally a cercaria which has been swimming for several hours may encyst in a container or on a slide. Encystment may occur in the presence of irritating substances, e.g., vital stains, which were used to study the penetration and cystogenous glands. Since the cercariæ do not ordinarily encyst in sea water, they were placed in dishes with various animals to discover the intermediate host or hosts in which they pass the next stage of their existence. A cercaria was observed to penetrate into the gill of *Mya arenaria*. It required about forty minutes to enter and encyst. In the process of penetration and encystment, the glands of the cercaria are emptied and a thin-walled cyst is formed. A specimen of *M. arenaria* was placed in a dish with many swimming cercariæ. On dissection of the mollusk four hours later, five cercariæ had lost their tails and were penetrating, one was found in the process of encysting, and twelve metacercariæ were recovered. Penetration and encystment was obtained in the mantle, gills, and foot of *Mya arenaria*, *Modiolus modiolus*, *Mytilus edulis*, *Cumingia tellinoides*, *Pecten irradians*, *Ensis directus*, and *Crepidula fornicata*. Cysts containing metacercariæ (Fig. 5) are spherical to oval, depending on relative pressure at the place of encystment, and measure from 0.14 to 0.19 mm. in diameter.

Development in the Final Host

Experimentally infected specimens of *M. arenaria* were fed to two white rats, three terns, *Sterna hirundo*, and five herring gulls, *Larus argentatus*. The rats did not pass eggs in the feces and both were negative when dissected three and eight weeks, respectively, after feeding. It appears, therefore, that the rat is not a suitable host for the development of the worms. The terns were killed, one day, three days, and twenty days after feeding. The metacercariæ excysted in the intestine of the terns and young worms were recovered from the first two birds examined. The third tern did not pass eggs in the feces and was negative when dissected twenty days after feeding. The gulls were killed, two, three, twelve, thirty-one, and forty-five days after feeding. Large numbers of worms in different stages of development were recovered from the first three gulls, about two hundred gravid worms were found in the fourth gull, and a single worm was present in the intestine of the last one dissected. A young worm removed after one day in the tern measured 0.6 mm. long and 0.154 mm. wide. The spines on the collar were 0.025 mm. in length; the acetabulum measured 0.1 mm. in diameter. The oral sucker was 0.056 mm. in diameter and the pharynx 0.04 mm. long by 0.028 mm. wide. A specimen (Fig. 6), recovered after three days' development in the gull, measured 0.74 mm. in length and 0.23 mm. in width. The collar spines measured 0.0294 mm. in length and 0.0077 mm. in width at the base; the acetabulum measured 0.14 mm. in diameter. The oral sucker was 0.06 mm. in diameter and the pharynx 0.04 mm. long by 0.035 mm. wide. Specimens (Fig. 7), recovered twelve days after they were fed to the gull, measure from 3 to 4 mm. in length. Although they are only about one-half grown, most of them contain eggs in the uterus. These studies have shown that the metacercariæ do not undergo development in the cyst and that the mollusk is actually little more than a transfer host. The metacercariæ were infective for birds three days after encystment and developed to maturity only in the intestine of *Larus argentatus*.

DISCUSSION

Miller and Northup (1926) examined 8,875 specimens of *Nassa obsoleta* from Quamquisset Harbor and found only a single species of echinostome cercaria which they described and named *Cercaria quissetensis*. In the present study over 10,000 specimens of *N. obsoleta* have been examined. All came from the Woods Hole region and about 1,000 of them from the same harbor where Miller and Northup obtained their material. A single species of echinostome cercaria was found which is so similar to that described by Miller and Northup that the two must be regarded as identical. The measurements agree closely and the differences between the descriptions are readily explainable. Miller and Northup reported only 27 cephalic spines. In the present study the exact number, 31, was first counted in the metacercaria and later confirmed in the cercaria.

In the preliminary papers (Stunkard 1934*a*, 1937), the adult worms recovered from the herring gull were regarded as specifically identical with those described by Linton (1928) from four species of Larus and from *Nycticorax nycticorax* and identified by him as *Himasthla elongata* (Mehlis). Although he referred his specimens to *H. elongata*, Linton noted certain differences between them and previous descriptions of that species. Linton's determination was questioned by Vogel (1933) and comparison of the present worms with the description of *Himasthla elongata* as given by Dietz (1910) shows such marked differences that they cannot be included in the same species. There are constant differences in the number and arrangement of the cephalic spines, size of organs, shape of testes, and in distribution of the vitellaria. In *H. elongata* the vitelline follicles are continuous, whereas in the species which I have studied these glands are interrupted at the levels of the testes. Since this species is distinct from *H. elongata*, the name given by Miller and Northup to its cercarial stage was adopted. The specimens identified as *H. elongata* by Linton were accordingly referred to *H. quissetensis*.

In the paper cited, Linton (1928) reported on several hundred specimens of *Himasthla* which had been collected at Woods Hole, Massachusetts, from four species of *Larus* and from *N. nycticorax*. He stated, "These distomes, while they vary considerably in size and proportions, appear to belong to the same species, and are in such close agreement with *H. elongata* that it seems best to refer them to that species in spite of the difference in the number of circum-oral spines.

H. elongata is characterized by having 29 circum-oral spines, of which the two which are situated at each angle of the oral disc are smaller than the others. In all of the specimens in which they could be distinctly seen in the Woods Hole material, the number of oral spines was found to be 31, arranged as shown in Figure 18." In this figure, 27 spines are situated in a single row with two between and behind the others at the ends of the row.

In addition to the specimens referred to *H. elongata*, Linton described two specimens from the white-winged scoter, *Oidemia deglandi*, as a new species which he named *Himasthla incisa*. In this species he reported that there are about 27 cephalic spines. The type specimen of *H. incisa* and representative specimens of the worms referred to *H. elongata* were deposited in the United States National Museum.

Lack of agreement between the number of cephalic spines on specimens referred by Linton to *H. elongata* and the number previously reported for that species, together with differences in the arrangement of the vitellaria in Linton's specimens and those of *H. quissetensis* have created a problem of specific determination. For comparison with material of *H. quissetensis*, Linton's specimens were borrowed from the U. S. National Museum. There were five slides, numbered 7921 to 7925 inclusive. The type specimen of *H. incisa* was mounted on slide No. 7925. This worm has 31 cephalic spines but the anterior part of the specimen is twisted and flattened, so their arrangement at the ends of the row is not symmetrical. The worm has heavy muscles and the cuticula bears prominent spines which extend posteriad as far as the level of the testes. The vitellaria are massive and extend along the sides of the body without interruption. Other specific features are presented in the measurements and figure of Linton. The specimen is clearly distinct from *H. quissetensis* and from all other described species. All of the other specimens had been identified as *H. elongata* by Linton. A single specimen from *Larus marinus* was mounted on slide 7923. It has 31 cephalic spines and the vitellaria are interrupted at the testicular zones. In both of these features as well as in total size and in the position and size of the individual organs it agrees with *H. quissetensis*. Consequently, I believe that this specimen should be assigned to that species. Nine specimens from *Larus argentatus*, which obviously belong to the same species, are mounted on slide 7921. They agree with Linton's figure 17 and all of them have 29 cephalic spines, 25 arranged in a single row with two between and behind the others at the tips of the collar. It is apparent that these specimens are distinct from the two described previously and their systematic position is doubtful. They may belong to *H. elongata* as Linton believed. A

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single specimen from *Nycticorax nycticorax* is mounted on slide 7924. It bears 29 cephalic spines and is probably identical with the worms from *L. argentatus* on slide 7921. Three specimens from *Larus delawarensis* are mounted on slide 7922. They have 29 cephalic spines, and although they are immature, the worms agree structurally with those on slides 7921 and 7924.

Subsequently, Professor Linton kindly sent additional material from his own collection. In a personal communication (April 11, 1938) he wrote, "Upon looking over my slides I find that I must have included a lot from the Herring Gull in my former report that were not *H. elongata*, as I find only 11, instead of 34 slides of that species." Among the specimens received from Professor Linton there is one which has 31 cephalic spines and interrupted vitellaria and which I regard as *H. quissetensis*. The others have 29 cephalic spines and are identical with the specimens on slides 7921 and 7924 from the U. S. National Museum collection.

It appears, therefore, that the specimens identified by Linton as *H. elongata* belong to two distinct species. Most of the worms agree with his figure 17 and they may be *H. elongata*. They have 29 cephalic spines and the measurements of twelve representative specimens are intermediate between or overlap the figures given by Dietz (1910) as characteristic for *H. elongata* and *H. militaris*. In my opinion the worms might with equal justification be referred to either of the two species. On the other hand, they may belong to neither. The second group of specimens referred by Linton to *H. elongata* have 31 cephalic spines and agree with the worms which I have described as *H. quissetensis*. The anterior end and cephalic spines of one of these specimens is shown in Linton's figure 18.

The precise status of the present and other species which have been referred to the genus *Himasthla* is doubtful. Until the amount of variation which naturally occurs in a species is determined, and its limits defined, final specific determination is impossible. In a related species, *Echinostoma revolutum*, Beaver (1937) has studied the amount and nature of variation. Certain of his observations are pertinent to the problem at hand. He found, p. 19, "The range then for mature worms is from 4 to 30 mm. in length. This size range is so great that a description of any one size is grossly inadequate for an accurate diagnosis of all other sizes, and a description by the ordinary methods is impractical when applied to worms having so great a range in size. This would not be true, however, if it were possible to give proportionate measurements applicable to all sizes which, as shown below, cannot be done. Practically every feature has been measured and plotted; and in no instance is the proportion between two organs or structures a constant throughout the series." He stated, p. 26, "Egg size varies with the age of the worm and is possibly somewhat altered by the host. The younger worms produce eggs with a very great range in size, and the average length is much greater than in more mature ones. The range in size for the eggs of the worms from all hosts was found to be 91 to 145 microns by 66 to 83 microns." Among other conclusions he reported, p. 65, "It is shown by this study that the cephalic spination is the most reliable character for diagnosis of the adult worm."

Sprehn (1932) listed *H. militaris* (Rudolphi, 1803) and *H. secunda* (Nicoll, 1906) as synonyms of *H. leptosoma* (Creplin, 1829) and Palombi (1934) admitted the probable identity of *H. leptosoma* and *H. secunda*. Since three of the four species of *Himasthla* which bear 29 cephalic spines were regarded as identical by Sprehn, one is led to question why the other one, *H. elongata* (Mehlis, 1831) was accepted as valid. There appears to be no better reason for retaining *H. elongata* than the other specific names which were dropped in synonymy. If the opinion of Sprehn is correct, the name of the species is *H. militaris* and not *H. leptosoma*.

With reference to *H. quissetensis*, it is not impossible that this species is identical with *H. alincia* Dietz, 1909, which also has 31 cephalic spines. The description of the latter species is based on a single specimen from *Tringa cinclus*, collected in Brazil. The figure of Dietz suggests the appearance of a worm which was dead before fixation and consequently much elongated. The worm is much more extended than the specimens which I have studied but the only obvious morphological difference between them is in the anterior limits of the vitellaria, which in *H. alincia* do not extend to the level of the cirrus sac.

A further case of specific identity is suggested between *H. quissetensis* and *H. muehlensi*, the species described by Vogel (1933) from man. A comparison of the specimens at hand with the description of *H. muehlensi* shows much similarity especially in the distribution of the vitellaria. The worms studied by Vogel were slightly larger and apparently contained one more spine in the cephalic coronet. But those specimens were dead and somewhat macerated; in none of them was the crown of spines complete and intact, and the absence of lateral crenations in the body wall may be correlated with the greater length of the extended specimens. Indeed, a specimen of *H. quissetensis* which was fixed in a moribund condition, is much extended and measures 14.2 mm. in length. The tissues had absorbed water and all the organs are larger than corresponding ones in well-fixed specimens. The testes were almost smooth. The differences between *H. quissetensis* and *H. muehlensi* in size and in dimensions of particular organs are not surprising in view of the difference in hosts and condition of material. Indeed, Vogel noted that species of *Himasthla* had previously been reported only from birds and he raised the question whether *H. muehlensi* may not normally be a bird parasite which occasionally is introduced into the human intestine where it may become mature but in which it is probably retained for only a short time. Consideration of this problem and of the possible difference in number of spines is facilitated by certain observations of Beaver (1937). He found in *Echinostoma revolutum* that large individuals may produce accessory collar spines. He stated that ordinarily the worms do not attain a definitive size, but continue to grow until they are expelled. From the study of specimens developed in six different mammalian and three different avian hosts he reported, p. 28, "As a rule the worms develop more rapidly in birds (Table 7, p. 77), become mature earlier and at a smaller size, and live a much shorter period." The data of Beaver show that differences induced in individuals of the same species when reared in avian and mammalian hosts are as great as those between *H. quissetensis* and *H. muehlensi.*

The first experimental demonstration of the life cycle in the genus *Himasthla* supports the opinion of Vogel that the human species, *H. muehlensi,* is acquired by eating raw or insufficiently cooked mollusks. Examination of large numbers of *Venus mercenaria,* purchased in the New York market, has failed, however, to disclose the metacercaria of this species. Whether or not it is distinct from *H. quissetensis* remains to be determined.

Results of the present study on *H. quissetensis* supplement those of Beaver on *Echinostoma revolutum.* All of the gulls were fed approximately the same number of metacercariæ and 100 to 200 worms were recovered from the intestine of each of the birds killed two, three, twelve and thirty-one days after experimental feeding. The worms collected after thirty-one days in the intestine may not have been fully mature, since the terminal half of the uterus contained very few eggs. The bird sacrificed forty-five days after ingestion of cysts had been passing large numbers of eggs in the feces but contained only a single specimen when killed. It appears that in the present species the worms do not persist in the intestine very long after they become sexually mature.

SUMMARY

Cercaria quissetensis Miller and Northup, 1926 has been restudied. It is the only echinostome cercaria which has been found in the

examination of over 18,000 specimens of *Nassa obsoleta* at Woods Hole. This cercaria encysts in the gills, mantle and foot of various mollusks. Metacercariæ were obtained experimentally in *Mya arenaria*, *Modiolus modiolus*, *Mytilus edulis*, *Cumingia tellinoides*, *Pecten irradians*, *Ensis directus*, and *Crepidula fornicata*. The metacercariæ are infective for birds three days after encystment and develop to maturity in the herring gull, *Larus argentatus*. Sexually mature specimens are identical with certain of those described by Linton (1928) as *Himasthla elongata* (Mehlis). Since there are important differences between the present specimens and *H. elongata*, they cannot be referred to that species and the name of the cercaria is adopted for them. Possible synonymy in the genus *Himasthla* is discussed.

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A METHOD FOR MAINTAINING A CONTINUOUS SUPPLY OF MARINE DIATOMS BY CULTURE[1]

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Culture techniques by means of which large supplies of unicellular organisms can be continually available are greatly in demand. This is especially true of unicellular plants, since they are convenient organisms for the study of photosynthetic and other metabolic processes.

The problem is essentially one of the maintenance of a growing population. So long as no factors develop which limit the rate of multiplication, increase in a culture or population is directly proportional to the number of organisms present. The growth of the population is logarithmic during the initial period. Some factor or factors in the environment, however, sooner or later lower the division rate. These factors may be limiting nutrient concentrations, formation of inhibitory excretory products, production of non-viable cells in the process of division, or, in the case of photosynthetic plants, limiting light intensity.

It was pointed out by Hjort, Jahn and Ottestad (1933), in a study of whaling in the antarctic, that the most advantageous way to exploit a population is to keep it at the level at which the greatest number of new organisms are produced in unit time. This paper presents an application of this principle to a culture method. After procuring the cell concentration at which the greatest daily yield is obtained the culture is maintained at this concentration. A volume of culture containing the quantity of cells equivalent to the daily increase is withdrawn each day and is replaced by an equal volume of fresh sea water enriched with nutrients.

We have used the marine diatom, *Nitzschia closterium*, which is an ideal organism for a study of culture technique. It is a small cell which is readily maintained in pure bacteria-free cultures, and which remains freely suspended in the sea water medium.

The culture medium used was that devised by Miquel for freshwater diatoms, and modified by Allen and Nelson (1910) for marine forms. We further modified it by the addition of $MgSO_4$ to the enrichment solutions.

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[1] Contribution No. 172 from the Woods Hole Oceanographic Institution.

Sea water was enriched by the addition of nutrients prepared as follows:

Solution A	KNO_3	20.2 grams/100 cc. water
Solution B	$Na_2HPO_4(12\ H_2O)$	4 grams
	$CaCl_2$	4 "
	$MgSO_4$	4 "
	$FeCl_3$ (melted)	2 cc.
	Conc. HCl	2 cc.
	dilute to	100 cc. with water

To each liter of sea water 0.55 cc. of solution A and 0.5 cc. of solution B were added. The reduction of the quantity of solution B from 1 cc. as used by Allen and Nelson to 0.5 cc. per liter was made in order to eliminate the precipitation of iron, magnesium and calcium phosphate which forms when larger quantities are used. The amount of solution A was adjusted to give the usual ratio of phosphate phosphorus to nitrate nitrogen in sea water. This ratio, as reported by Redfield (1934), is about twenty atoms of nitrogen to one of phosphorus.

In the culture of unicellular plants it is difficult to obtain sufficient light intensity without undesirable heating. Neon tubes obviate this difficulty since they radiate very little heat, and the emission is close to the optimum for chlorophyll synthesis. The apparatus finally devised consists of a U-shaped neon tube which is surrounded by a glass water-jacket 8 cm. in diameter. This is placed in the axis of a large cylindrical museum jar 21.3 cm. in diameter by 90 cm. in height. The culture occupies the space between the two cylinders. The neon tube is completely surrounded by the culture and little light is lost except that which passes through the medium without being absorbed. By regulation of the flow of water through the water jacket the temperature in the culture can be controlled. Air containing 5 per cent CO_2 is bubbled through the culture to maintain a large source of available carbon, and to keep the cells from settling. Nutrient solutions may be added to the culture from time to time by means of a dropping funnel.

In flask cultures of *Nitzschia closterium* in the above medium the growth is limited to 2×10^9 cells per liter by the complete exhaustion of nutrients. In the large culture nutrients are added when necessary and ten times the maximum cell concentration produced in flask cultures can be obtained. Every time a number of cells sufficient to exhaust the nutrients is formed the appropriate amounts of the nutrient solutions must be added. The necessity for enrichment may be determined by following the cell count or may be checked by periodical chemical analyses of the medium.

In Table I the data for the growth of a typical culture are presented.

TABLE I

Growth of a culture of twenty-six liters of Nitzschia closterium with regular enrichment of the medium.

Time	Cell count	Division rate (k) per day	Daily yield
days	*cells/liter*		*cells/liter*
0	0.004×10^9	—	—
2	0.043 "	1.19	0.02×10^9
3	0.140 "	1.15	0.10 "
5	0.52 "	0.655	0.19 "
6	1.04 "	0.530	0.52 "
7	2.10 "	0.530	1.06 "
9	3.58 "	0.27	0.75 "
11	8.10 "	0.40	2.25 "
13	11.5 "	0.18	1.7 "
14	12.8 "	0.11	1.3 "
15	13.8 "	0.07	1.0 "

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The division rate per cell is expressed by the velocity coefficient of the rate of growth, k. It is obtained from the equation

$$k = \frac{1}{t_2 - t_1} \cdot \ln \frac{C_{t_2}}{C_{t_1}}$$

in which C_{t_2} and C_{t_1} are respectively the cell concentrations at the end and beginning of the period under consideration. The division rate per cell remained approximately constant for the first three days of the culture period, after which it declined steadily.

The daily yield depends upon both the division rate and the cell concentration. It increased to a maximum on the eleventh day and then decreased. It is apparent that in order to obtain the maximum production of the culture it should be maintained at the cell concentration giving the optimum daily yield.

In Table II are given data procured by periodically reducing the cell concentration to the optimum by removal of the daily yield and subsequent dilution to the original volume. The values for the daily yield given in column five show that the production can be steadily maintained at its maximum. At the optimum cell concentration of 8×10^9 cells per liter 28 per cent of the total culture can be removed daily, giving a daily yield of about 2.4×10^9 cells per liter.

The total yield of a culture depends, of course, on its volume. The apparatus used in obtaining these data contained 26 liters, giving a total yield of about 62 × 10^9 cells per day. The dry weight of this quantity is approximately one gram. For the fifteen-day growth period of the culture (Table I) the average daily yield for the total period is only 24 × 10^9 cells per day. This value is only about one-third of that obtained by the renewal method after optimal conditions have been established and no continuous supply is obtained.

TABLE II

Yield of a culture of Nitzschia closterium maintained continuously at the optimal concentration for production.

Initial count	Final count	Duration of growth period	Division rate (k) per day	Daily yield
cells/liter	*cells/liter*	*hours*		*cells/liter*
8.84 × 10^9	11.36 × 10^9	25	0.243	2.42 × 10^9
7.70 "	11.15 "	41	0.217	2.02 "
8.10 "	12.92 "	45	0.250	2.57 "

This culture method was originally undertaken in an attempt to obtain diatoms for chemical analyses in quantities ranging up to a kilogram or more of dry material. The time required for producing such a quantity by this method could be reduced by increasing the number of units. A direct enlargement of the culture chamber is not desirable, since an increase in thickness of the culture would make the light intensity a serious limiting factor. A larger container could be used if it contained several light sources. A method for obtaining large quantities of diatoms directly from the sea has been described by Clarke (1937). Allen (1938) has recently criticized Clarke's method as being unnecessarily elaborate, but she does not appear to appreciate the magnitude of the quantities required, nor our need for a method applicable under those conditions of abundance frequently encountered in the sea.

The culture method described above has the advantage, not obtainable with natural collections, of producing a continuous supply of cells in a pure state. The culture can, moreover, be grown under various controlled environmental conditions and an ample quantity of material is produced daily for a variety of chemical and physiological tests. It is believed that a similar method can be applied advantageously to many other unicellular organisms required for physiological research.

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PARTHENOGENETIC MEROGONY OR DEVELOPMENT WITHOUT NUCLEI OF THE EGGS OF SEA URCHINS FROM NAPLES

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In a previous paper (1936) it was shown that non-nucleate parts of the eggs of *Arbacia punctulata*, obtained by centrifugal force, can be artificially activated and by successive cleavages they reach the blastula stage. This I have termed parthenogenetic merogony since it is a combination of parthenogenesis, in which the male nucleus is lacking, and merogony, in which the female nucleus is lacking; in parthenogenetic merogony both male and female nuclei are lacking, and the egg develops without any nuclei. In view of the fact that the non-nucleate halves of *Arbacia punctulata*, obtained by centrifugal force, are densely packed with pigment granules which apparently interfere somewhat with the formation of typical cell boundaries, even after fertilization, it seemed possible that further development of parthenogenetic merogones might be obtained with other species of sea urchin. Furthermore, the presence of mitochondria in the non-nucleate halves of certain species presented a means of testing the possibility that these granules might be helpful in development without nuclei. An investigation was carried out at Naples in the spring of 1937 and the results of the study are presented in this paper. Some observations on parthenogenetic and merogonic development are included, to serve as a basis for comparison of eggs without any nucleus (parthenogenetic merogones) with similar eggs and egg fractions having one nucleus, female or male (= "haplones"). A study has previously been made (1933) of the development of the centrifuged whole eggs and their nucleate fractions after fertilization, that is with two nuclei, both female and male (= "diplones"). Some observations on the effect of activating agents on immature eggs are also included since these bear on the general subject.

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[1] I am very grateful to Dr. Reinhard Dohrn for his interest and kindness during my stay at the Stazione Zoologica in Naples and to the Rockefeller Institute for the use of the Jacques Loeb Memorial Table. I wish also to acknowledge with thanks a grant from the Penrose Fund of the American Philosophical Society which has greatly facilitated my work.

MATERIAL AND METHOD

The four species of sea urchins commonly occurring at Naples are *Arbacia pustulosa*, *Sphaerechinus granularis*, *Paracentrotus* (*Strongylocentrotus*) *lividus* and *Parechinus* (*Echinus*) *microtuberculatus*. The eggs of all these species can be broken apart by centrifugal force and these parts can be fertilized and will develop (1933). The nucleus always goes to the light pole, so that the heavy half of the egg is always non-nucleate. With further centrifuging, the halves can be broken into quarters. The non-nucleate fractions of the eggs of all four species can be artificially activated. The egg of *Parechinus microtuberculatus* proved the best one for the investigation since it breaks apart easily (thus avoiding heating while on the centrifuge) and the very clear protoplasm renders all phenomena easily visible in the living egg.

The most satisfactory parthenogenetic agent was the same as was used for *Arbacia punctulata*, i.e., sea water concentrated to about half its volume, or NaCl added to the sea water to bring it to a similar hypertonicity. After immersion for 10–20 minutes, the eggs were returned to sea water. Other methods were tried, especially Loeb's double method (butyric acid and a hypertonic solution) with less favorable results. One difficulty in the investigation has been to obtain a standard concentration and a definite period of immersion in the hypertonic solution owing to the extreme variability in reaction of different batches of eggs of the same species. Such has been the experience also of other investigators in obtaining parthenogenesis of entire eggs.

STRATIFICATION BY CENTRIFUGAL FORCE

When the eggs of any of the four species are centrifuged, the nucleus always goes to the light pole under the oil cap. This is invariable in mature unfertilized eggs. The stratification of materials, however, is different in the different species, thus causing a difference in granular content of the non-nucleate fractions of the different species; these fractions also differ in size in comparison with their corresponding nucleate fractions. The stratification of the four species is shown in Photographs 1–13.

The normal egg of *Arbacia pustulosa* is exactly like that of *Arbacia punctulata* except that it is a little larger (79 μ as vs. 74 μ) and more heavily pigmented; and it stratifies similarly. The stratification of *Arbacia pustulosa* (Photograph 1) is:—oil at the light pole, then a clear layer, then a band of mitochondria (small granules which stain purple with methyl green), then yolk granules, and then red pigment granules at the heavy pole. In the other three species pigment granules are lacking, except for the faint (occasionally quite reddish) band encircling the *Paracentrotus* egg, which seems to be a definite structure not moved by the centrifugal forces used in my experiments. This is probably due to the fact that the pigment granules forming the band are embedded in the cortical protoplasm which is generally conceded to have a higher viscosity than the interior. The egg of *Arbacia pustulosa* breaks apart in 3 minutes at 10,000 × g into two almost equal halves, the non-nucleate half containing only yolk and pigment and no mitochondria (Photograph 2). With further centrifuging, the halves can be broken into quarters (Photograph 3).

The normal egg of *Sphaerechinus granularis* averages 98 μ in diameter. The stratification under centrifugal force is similar to that of *Arbacia* except for lack of pigment granules; i.e., oil, clear layer, mitochondria and yolk (Photograph 4). There is frequently a clear layer also at the centrifugal pole below the yolk (Photograph 5). The egg breaks apart at 10,000 × g in 5 minutes (Photograph 6). The non-nucleate fraction is much larger than the lighter nucleate fraction and contains yellowish yolk granules and a few mitochondria. With further centrifuging both half-eggs can be broken into quarters.

The eggs of the other two species differ from those of *Arbacia* and *Sphaerechinus* in having the mitochondria heavier than the yolk granules. The egg of *Paracentrotus lividus* has an average diameter of 90 μ. The usual stratification under centrifugal force is:—oil, clear

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PHOTOGRAPHS 1–13

Stratification and Breaking of Eggs

The photographs were all taken of the living eggs with a Leica camera. Photographs 1, 4, 5, 6, 7, 12 were taken of eggs in a centrifuge microscope slide immediately after being rotated on the centrifuge microscope, and all with the same magnification, so that the sizes of the eggs as pictured are comparative.

PHOTOGRAPH 1. *Arbacia pustulosa*—Stratification.

PHOTOGRAPH 2. *Arbacia pustulosa*—Stratified whole and half-eggs.

PHOTOGRAPH 3. *Arbacia pustulosa*—Red half and its two quarters.

PHOTOGRAPH 4. *Sphaerechinus granularis*—Stratification.

PHOTOGRAPH 5. *Sphaerechinus granularis*—Stratification showing clear layer at bottom of eggs.

PHOTOGRAPH 6. *Sphaerechinus granularis*—Breaking apart.

PHOTOGRAPH 7. *Paracentrotus lividus*—Usual stratification.

PHOTOGRAPH 8. *Paracentrotus lividus*—Stratification with clear layer at bottom of egg.

PHOTOGRAPH 9. *Paracentrotus lividus*—No clear layer at top of egg (Like *Parechinus*).

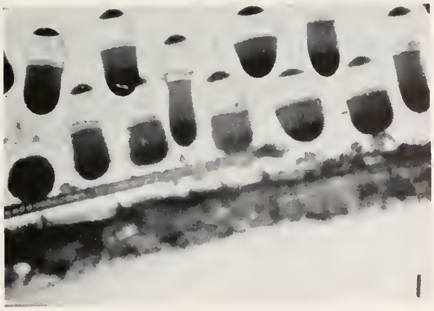
PHOTOGRAPH 10. *Paracentrotus lividus*—Breaking into large upper and small lower halves.

PHOTOGRAPH 11. *Paracentrotus lividus*—Breaking into small upper and large lower halves.

PHOTOGRAPH 12. *Parechinus microtuberculatus*—Stratification.

PHOTOGRAPH 13. *Parechinus microtuberculatus*—Breaking apart.

PLATE I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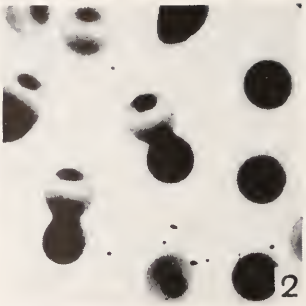
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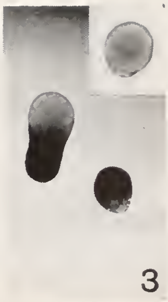
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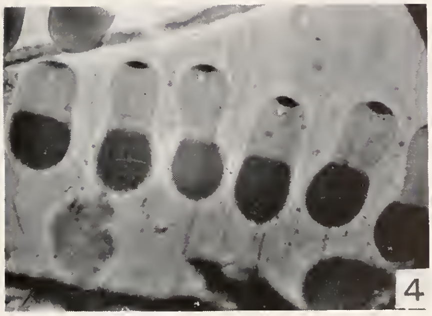
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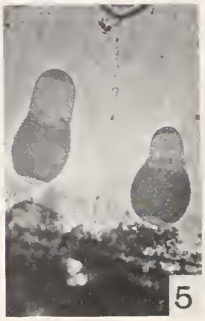
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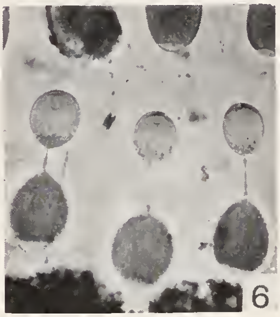
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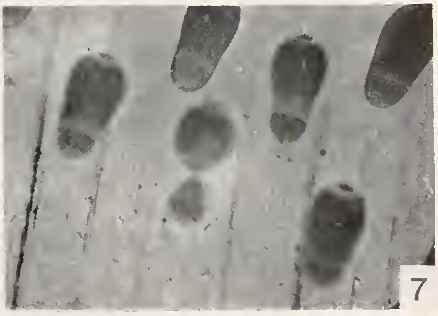
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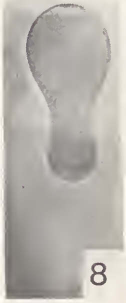
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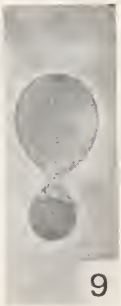
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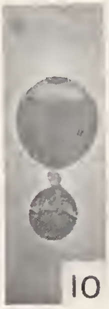
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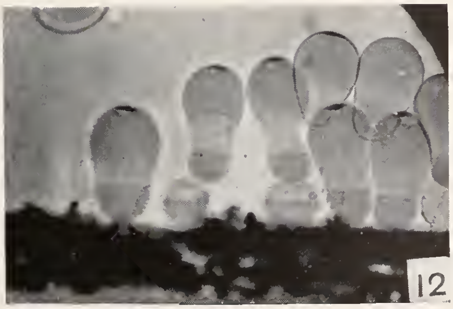
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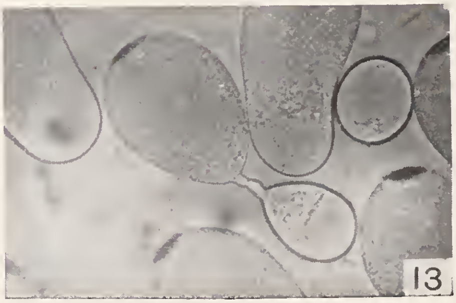
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layer, yolk, clear layer and mitochondria (Photograph 7). There is, however, some variation in the position of the clear layer; in some eggs there is no clear layer between the yolk and mitochondria; in some eggs there is a clear zone below the mitochondria (Photograph 8). And sometimes there is no clear layer below the oil (Photograph 9); in this case the stratification is like that of *Parechinus* (Cf. Photographs 9 and 12). The clear substratum is apparently, therefore, not uniform, but must be of graded density throughout its extent in the centrifuged egg. It takes 30 minutes at 10,000 × g to break these eggs apart; they are much more difficult to break apart than the eggs of the other species. There are two types of breaking. The egg may break into a larger nucleate fraction and a small non-nucleate sphere, as the majority of eggs did in the springs of 1933, 1934 and 1937 (Photograph 10) or they may break into a smaller nucleate fraction and a large non-nucleate fraction as the majority of eggs did in 1932 (Photograph 11) (E. B. Harvey, 1933). The two types must be due to something inherent in the egg as they may occur in eggs from the same female centrifuged together in the same tube, although any set of eggs tends to be either of the one type or the other. A similar peculiarity has been noted previously for *Arbacia punctulata* in unusual batches of eggs (1936). In either case, the non-nucleate sphere of *Paracentrotus* contains all the mitochondria and some of the yolk granules.

The egg of *Parechinus microtuberculatus* is the largest of the four species; its average diameter is 102 μ. The typical stratification is:—oil, yolk granules, clear layer, mitochondria (Photograph 12). The nucleus, therefore, lies typically among granules at the centripetal pole and not in a clear layer as in the eggs of the other species of sea urchins and of most other eggs. There is occasionally, however, also a clear layer under the oil in which case this egg resembles the typical *Paracentrotus* egg. The egg breaks apart in 8 minutes at 10,000 × g, and with further centrifuging the lower half can be broken into quarters. The non-nucleate halves are much smaller than the nucleate, and contain mitochondria and practically no other granules (Photograph 13).

We see, then, that the eggs of the four species of sea urchin under consideration stratify differently, and that therefore the non-nucleate fractions contain different kinds of granules. In the non-nucleate halves of *Arbacia*, pigment granules are present, in the other three species, they are not. In *Sphaerechinus*, *Paracentrotus* and *Parechinus*, mitochondrial granules are present, in *Arbacia*, they are not. In *Parechinus* there are practically no yolk granules, but these are present in the non-nucleate halves of the other three species. The clear protoplasm which is the matrix in which granules are suspended is, of course, present in all the non-nucleate halves, in greater or less amount, and appears as a definite layer when free of granules, as is typical for *Parechinus*.

ARBACIA PUSTULOSA

Parthenogenetic Merogones

The non-nucleate red halves, yolk quarters and pigment quarters of *Arbacia pustulosa* have been activated artificially (= parthenogenetic merogones). They throw off a fertilization membrane closely adhering to the surface and form a thick ectoplasmic layer, as is characteristic of the normal fertilized egg of this species. A large monaster appears in many of the fractions about two hours after activation, succeeded in another hour or so by an amphiaster; these are usually visible only in the yolk quarters and the less pigmented part of the red halves. After another hour, a cleavage plane may come in between the two asters. This has been observed to come in chiefly in the long axis of the elongate red half-eggs and to extend only to the pigmented area. Some of the red halves have reached by successive cleavages a 16-cell stage with good cell boundaries. Photographs 14–17 are successive photographs of the same two eggs. In many red halves, cell boundaries do not come in, but the egg may be literally peppered with small asters (Photograph 18). In many of the red half-eggs, a cleavage takes place at the pigmented end (Photographs 19, 20). In the yolk quarters which are usually spherical, the first cleavage plane divides the fraction equatorially or nearly so (Photograph 21). No further development has been obtained of the parthenogenetic merogones of *Arbacia pustulosa*, but it seems likely that with a more extensive study, blastulæ could be obtained similar to those of *Arbacia punctulata*.

Haplones

The term "*haplone*" is used to include eggs which develop with only one nucleus whether female (= parthenogenetic) or male (= merogonic, i.e. a fertilized egg fragment from which the female nucleus has been removed) and therefore containing a haploid set of chromosomes. The normal fertilized egg, containing both male and female nuclei and therefore a diploid set of chromosomes, is termed a "*diplone*."

The normal egg of *Arbacia pustulosa* may be activated by parthenogenetic agents, and also the whole egg after it has been centrifuged, as well as the white half which contains the nucleus. They all throw off a closely adherent fertilization membrane characteristic of the normal fertilized egg. The cleavage is similar to that of the fertilized egg except that it takes place at a slower rate. The first cleavage of the artificially activated egg, both normal and centrifuged, takes place only after 3 hours (at 20° C.), often much longer, whereas that of the fertilized egg takes place in $1\frac{1}{3}$ hours. The two-cell stage of the elongate centrifuged egg, both fertilized and parthenogenetic, is similar to that of *Arbacia punctulata;* the first cleavage plane usually comes in across the short axis of the egg, in the less dense granular area, dividing the egg into a smaller cell with few granules and a larger pigmented cell (Photograph 22). The first cleavage plane of the spherical white half, both fertilized and parthenogenetic, is equatorial (Photograph 23). Parthenogenetic whole eggs, both normal and centrifuged, and parthenogenetic white halves have given swimming blastulæ, but were carried no further.

The non-nucleate halves and quarters when fertilized (= fertilized merogones) have sometimes cleaved quite regularly, when spherical, but at a slow rate (Photograph 24); but often, as in *Arbacia punctulata*, cell boundaries fail to come in, giving rise to multi-nucleate eggs. Nuclei are often plainly visible in these fertilized merogones (Photograph 25). They contrast strikingly in this respect with the parthenogenetic merogones in which asters may be present but not nuclei. A few swimmers have been obtained but many of the fertilized merogones fail to develop.

PARECHINUS MICROTUBERCULATUS

Parthenogenetic Merogones

The non-nucleate halves of *Parechinus microtuberculatus* consist of mitochondrial granules and clear layer (Photograph 26). When they

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PHOTOGRAPHS 14–25

Arbacia pustulosa

PHOTOGRAPHS 14–21. Parthenogenetic merogones. Photographs 22–25. Haplones.

PHOTOGRAPH 14. Two red half-eggs, 4½ hours after activation. Two asters were plainly visible in the egg at left.

PHOTOGRAPH 15. Same, 5 minutes later. Cleavage between the two asters.

PHOTOGRAPH 16. Same, 1 hour later (5½ hours after activation). Note cleavage plane appearing in egg at right.

PHOTOGRAPH 17. Same, 1/2 hour later (6 hours after activation).

PHOTOGRAPH 18. Red half-egg, peppered with asters (7½ hours after activation).

PHOTOGRAPH 19. Red half-egg, 5¼ hours after activation.

PHOTOGRAPH 20. Similar red half-egg, 22 hours after activation.

PHOTOGRAPH 21. Yolk quarter, 4½ hours after activation. The cleavage plane was observed to come in between two large asters.

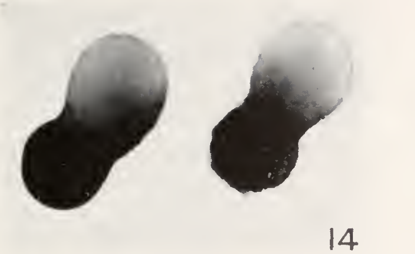
PHOTOGRAPH 22. Whole egg centrifuged, then activated, 6 hours after activation.

PHOTOGRAPH 23. White half-egg, 6 hours after activation.

PHOTOGRAPH 24. Fertilized red half, 5¼ hours after fertilization.

PHOTOGRAPH 25. Fertilized yolk quarter, 9½ hours after fertilization. Observe nuclei in the cells.

PLATE II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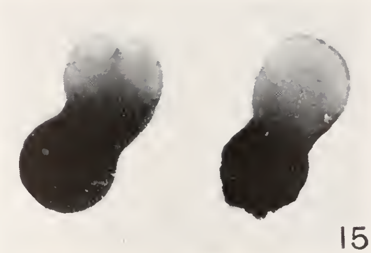
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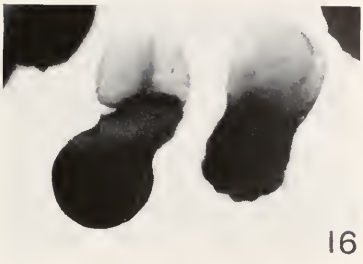
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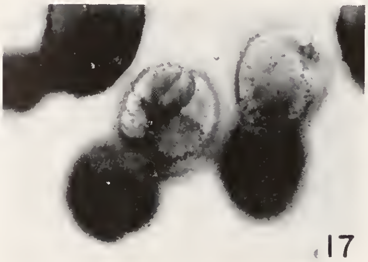
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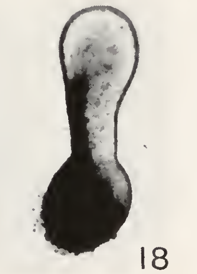
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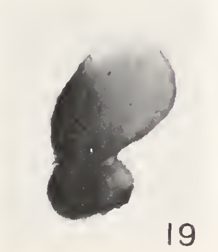
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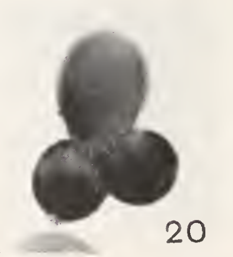
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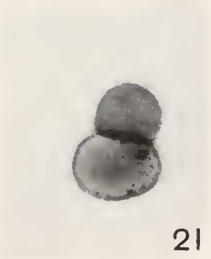
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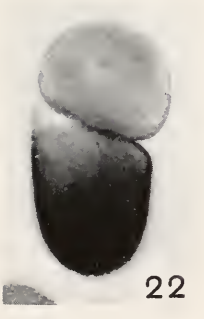
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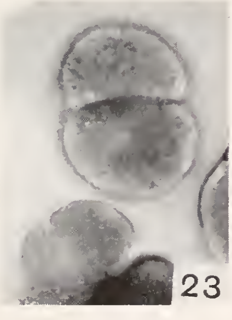
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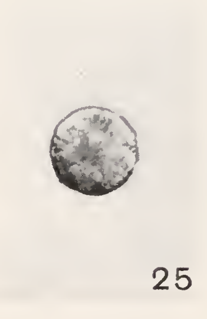
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are activated, they throw off fertilization membranes widely separated from the surface of the egg, leaving a large perivitelline space just as is characteristic of the normal fertilized egg of this species (Photograph 27). The membranes are frequently thrown off while in the hypertonic solution and the surface of the egg then becomes irregular or crenate (Photograph 28). On return to sea water, these eggs swell and the perivitelline space is often completely obliterated; the fertilization membrane is frequently broken by further swelling. A large monaster forms three or four hours after activation and after another hour or so there may be two, three or more asters. Cleavage planes have been observed coming in around the asters, resulting in two-, three-, or four-cell stages (Photographs 29, 30, 31). No regular succession of cleavages has been observed, such as occur in *Arbacia*. The egg seems somewhat labile and cleavage planes appear and disappear. These eggs later break up into a number of small cells, becoming progressively smaller and more numerous, often while still within the fertilization membrane, resembling a blastula. Photographs 31, 32 and 33 are successive stages of the same egg showing the breaking up, following normal cleavage planes (Photograph 31) which had been observed to come in between three large asters. Photographs 34, 35 and 36 are successive stages of another egg. Many of these blastulæ occur as well as other blastulæ in which there are no cell boundaries; these may be filled with asters. The blastulæ emerge from the fertilization membranes in quite typical fashion on the day after activation, leaving the ruptured empty membranes (Photograph 37) and they may live for several days. The characteristic breaking up of the non-nucleate half-egg might be looked upon as stages in degeneration rather than as stages in development. But an exactly similar process takes place also

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PHOTOGRAPHS 26–37

Parechinus microtuberculatus. Parthenogenetic merogones.

PHOTOGRAPH 26. Non-nucleate lower halves, just broken off by centrifuging.

PHOTOGRAPH 27. Ten minutes after activation, to show fertilization membranes.

PHOTOGRAPH 28. Non-nucleate halves still in hypertonic sea water for 1/2 hour.

PHOTOGRAPH 29. Beginning two-cell stage, still in hypertonic sea water for three hours.

PHOTOGRAPH 30. Two-cell stage, seven hours after activation.

PHOTOGRAPH 31. Three-cell stage, four hours after activation; three large asters were plainly visible but do not show in the photograph.

PHOTOGRAPH 32. Same egg as 31, five hours later.

PHOTOGRAPH 33. Same egg, one hour later (ten hours after activation).

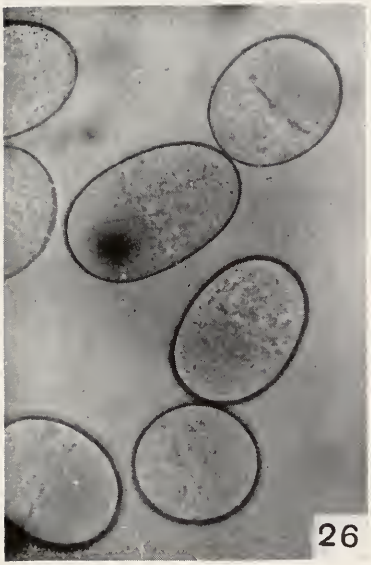
PHOTOGRAPH 34. Another egg, twenty-two hours after activation.

PHOTOGRAPH 35. Same egg, five hours later.

PHOTOGRAPH 36. Same egg, six hours later (thirty-three hours after activation).

PHOTOGRAPH 37. Non-nucleate egg emerging from fertilization membrane, twenty hours after activation.

PLATE III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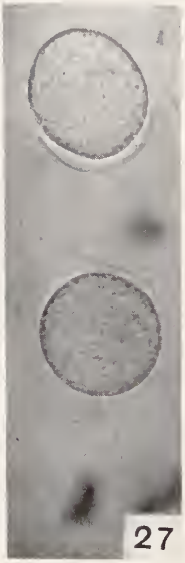
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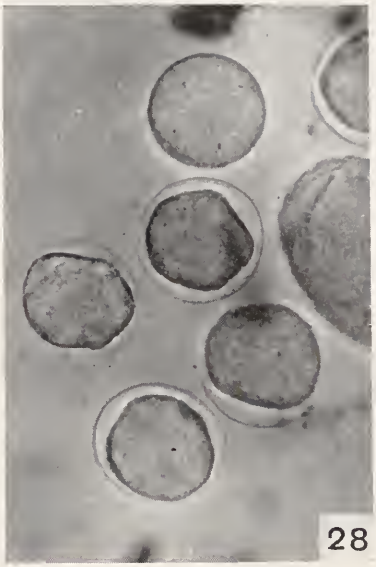
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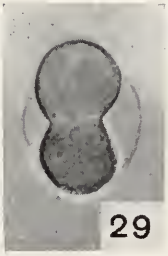
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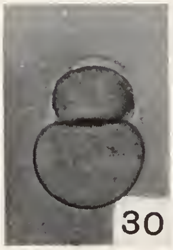
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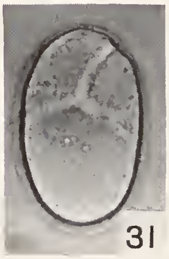
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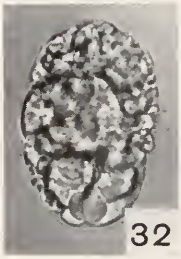
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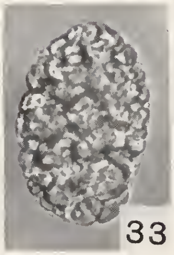
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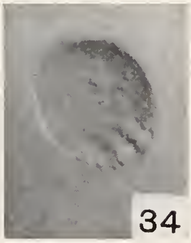
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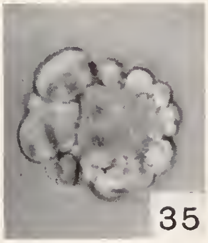
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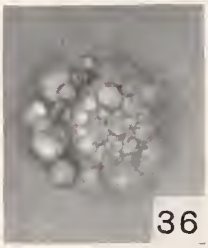
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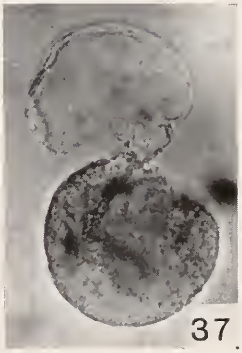
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in the whole egg leading to normal blastulæ as described below. The similarity may be readily seen by comparing Photographs 34, 35 and 36 with Photographs 39, 40 and 42.

Haplones

Normal uncentrifuged eggs of *Parechinus microtuberculatus* were treated with hypertonic sea water for twenty-five minutes, then transferred to sea water. Fertilization membranes formed and after some hours the nuclei enlarged as they normally do previous to breaking down. In Photograph 38, taken seven and a half hours after activation, the enlarged nuclei may be seen in the two eggs to the left, and in the right-hand egg, the nuclear membrane has just broken (it was observed to break) and the cell has become somewhat amoeboid. Fifteen minutes later, this egg has begun to break up into a number of irregular cells (Photograph 39) and the process has continued further in Photograph 40, taken fifteen minutes later. The process continues as is shown in Photographs 41 and 42, until a blastula is formed, often quite normal in appearance, consisting of many cells of the same size. After some hours the blastulæ break through the fertilization membranes and become free-swimming, with cilia. Some of these seem quite regular and normal but some are abnormal consisting of cells of varying sizes. Many normal gastrulæ later developed, presumably (not actually observed) from the blastulæ which had developed in this irregular fashion (Photograph 43). A comparison of Photographs 34 with 39, 35 with 40, and 36 with 42 will show the similarity of the breaking up of the non-nucleate egg with the process in the nucleate parthenogenetic egg which gives rise to some quite normal-looking swimmers.

Some centrifuged whole eggs treated with the parthenogenetic agent cleaved similarly to such eggs after fertilization. In one set of

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PHOTOGRAPHS 38–46

Parechinus microtuberculatus. Haplones.

PHOTOGRAPH 38. Normal eggs, 7½ hours after activation. Nuclear membrane had just broken in egg to right.

PHOTOGRAPH 39. Same eggs, 25 minutes later; egg breaking up irregularly.

PHOTOGRAPH 40. Same eggs, 15 minutes later.

PHOTOGRAPH 41. Similar eggs, 7½ hours after activation.

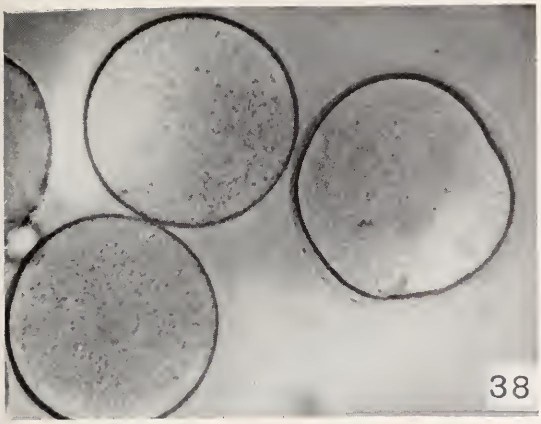
PHOTOGRAPH 42. Similar eggs, 7½ hours after activation. Note perfect blastula.

PHOTOGRAPH 43. Similar eggs, 24 hours after activation. Note perfect gastrula.

PHOTOGRAPH 44. Centrifuged egg; cell division, 15 minutes after activation, while nucleus is still intact.

PHOTOGRAPH 45. Similar eggs, 5 hours later; upper part cytolyzed.

PHOTOGRAPH 46. Fertilized merogone, 2 hours after fertilization.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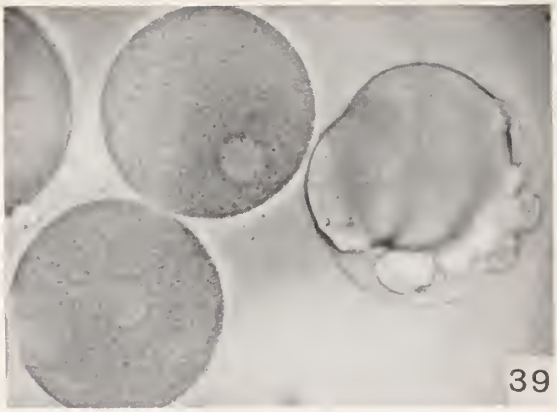
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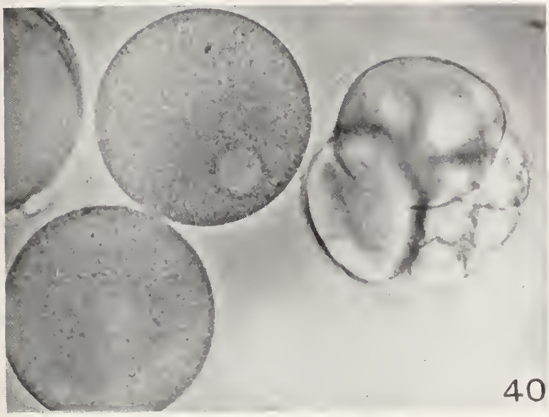
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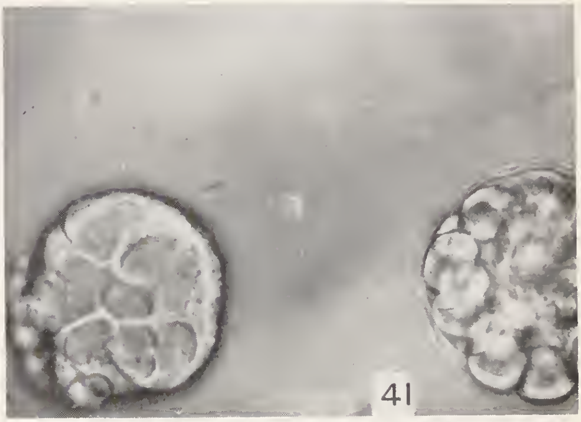
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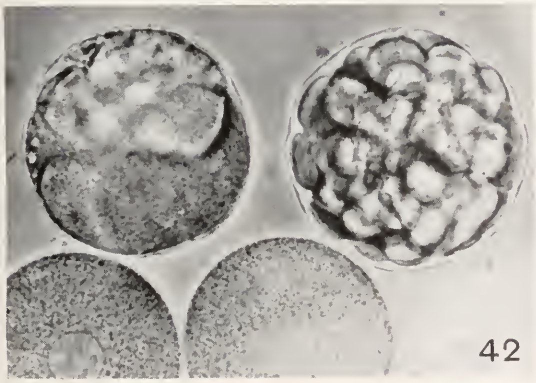
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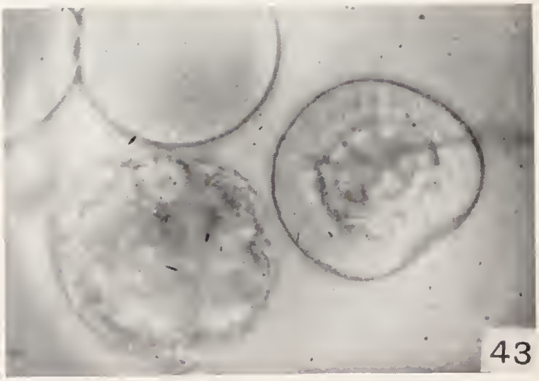
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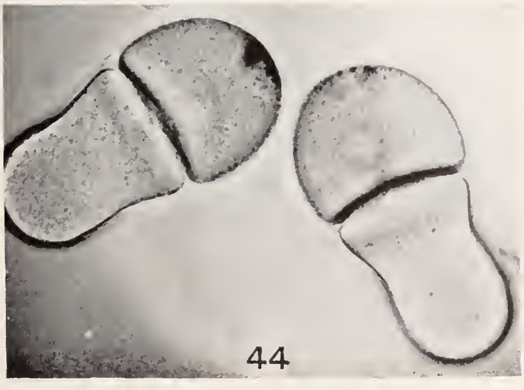
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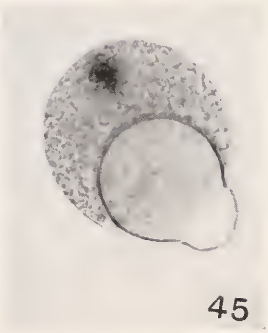
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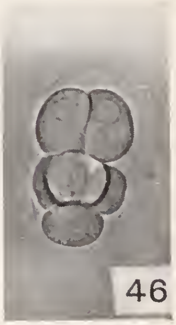
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parthenogenetic centrifuged eggs, however, a cleavage plane came in fifteen minutes after activation while the nucleus was still intact (Photograph 44). This came across the short axis of the egg, producing the same two-cell stage characteristic of the elongate *Arbacia* egg, both fertilized and parthenogenetic (Cf. Photograph 44 with 22, and with 30, 31 and 36 of my 1936 paper). This is another instance of cleavage planes coming in independently of any nuclear changes and emphasizes the fact that the cleavage of an egg and the nuclear changes usually accompanying it are quite separate phenomena. Later on, the upper part of these eggs cytolyzed, leaving the lower part with mitochondrial granules, intact (Photograph 45).

The fertilized merogones in many cases cleaved quite regularly. Photograph 46 is an eight-cell stage showing nuclei.

PARACENTROTUS LIVIDUS

Parthenogenetic Merogones

The development of the non-nucleate fractions of *Paracentrotus* after activation is exactly like that of *Parechinus* described above. The fertilization membrane is thrown off, leaving a large perivitelline space characteristic of the normal fertilized egg of this species. A conspicuous large aster is formed (Photograph 47) followed by two or more smaller asters. A few early cleavages have been observed (Photograph 48) but the cleavage planes come and go and then the egg breaks up into a number of cells becoming progressively smaller and more numerous, just as in *Parechinus*, giving rise to a blastula-like embryo (Photographs 49, 50).

PHOTOGRAPHS 47–58

PHOTOGRAPHS 47–51. *Paracentrotus lividus.* Photographs 52–58. *Sphaerechinus granularis.*

PHOTOGRAPH 47. *Paracentrotus lividus.* Non-nucleate half, 1½ hours after activation. Note large monaster.

PHOTOGRAPH 48. Beginning 2-cell stage, 4 hours after activation.

PHOTOGRAPH 49. Same egg as 47, 2½ hours after activation; egg fragmenting.

PHOTOGRAPH 50. Same egg 1/2 hour later.

PHOTOGRAPH 51. Parthenogenetic centrifuged whole egg, tripartite. Note aster in the stalk and many asters in upper part.

PHOTOGRAPH 52. *Sphaerechinus granularis.* Non-nucleate half, 2 hours after activation.

PHOTOGRAPH 53. Same egg, 3 hours later.

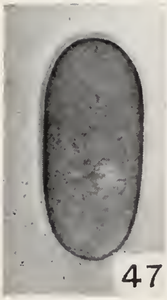
PHOTOGRAPH 54. Same egg, 1/2 hour later.

PHOTOGRAPH 55. Same egg, 1 hour later.

PHOTOGRAPH 56. Parthenogenetic centrifuged whole egg, 5 hours after activation.

PHOTOGRAPHS 57, 58. Cleavage of parthenogenetic centrifuged whole eggs while nucleus is still intact; 1 hour after activation.

PLATE V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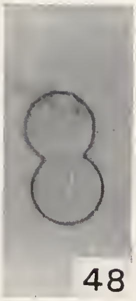
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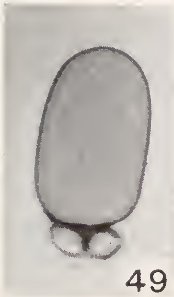
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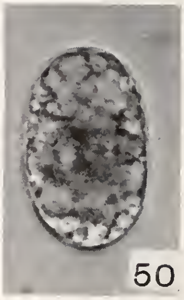
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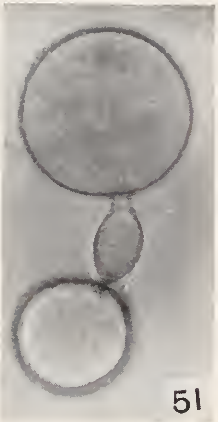
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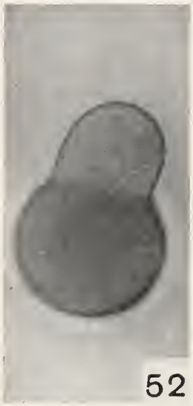
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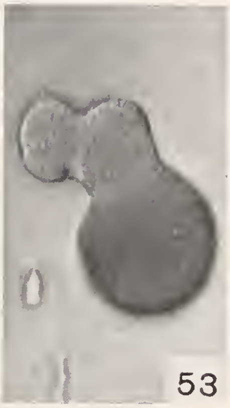
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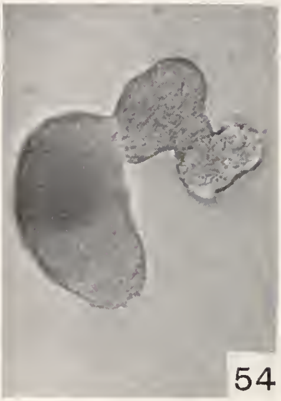
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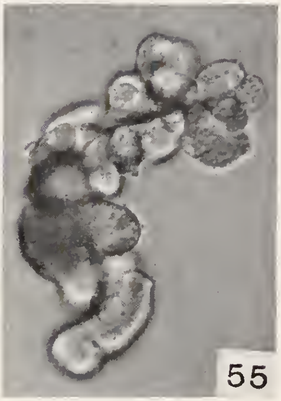
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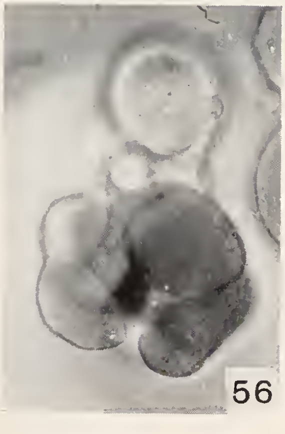
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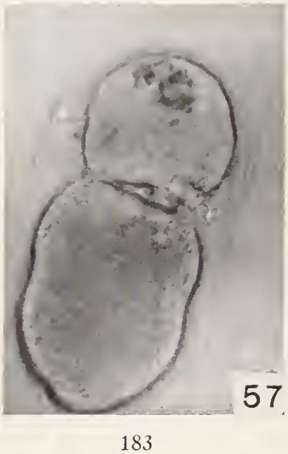
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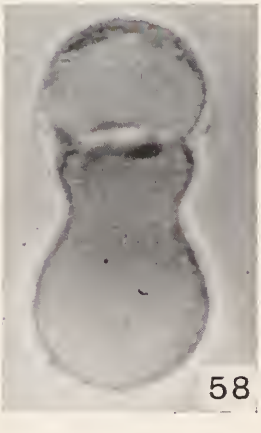
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Haplones

In the normal egg and in the centrifuged whole egg treated with hypertonic solutions, asters frequently appear in the protoplasm while the nucleus is still intact. Attention has previously been called (1933) to the peculiar tripartite eggs which develop after fertilization of a centrifuged egg, giving rise to three embryos. The middle portion of these eggs is simply the thickened stalk between the two half-eggs when incompletely broken apart. In a similar parthenogenetic egg, the fertilization membrane forms also around the stalk and a monaster appears in the stalk, even before the nucleus in the upper half has broken down. This is shown in Photograph 51 in which the upper part is filled with many asters (light areas in the photograph). Later on, the upper nucleate part of the centrifuged egg becomes cytolyzed, while the lower part may remain intact and even cleave. The cleavage of the lower part may also take place while the nucleus is intact in the upper part.

Swimmers have been obtained from parthenogenetic normal eggs but not from centrifuged whole eggs or the white nucleate halves after parthenogenetic reagents.

Good cleavages of the fertilized merogones have been obtained. Owing to the long period of centrifuging (thirty minutes) necessary for breaking apart the *Paracentrotus* egg, and the consequent heating up of the machine and the eggs, investigation of this species has been limited.

SPHAERECHINUS GRANULARIS

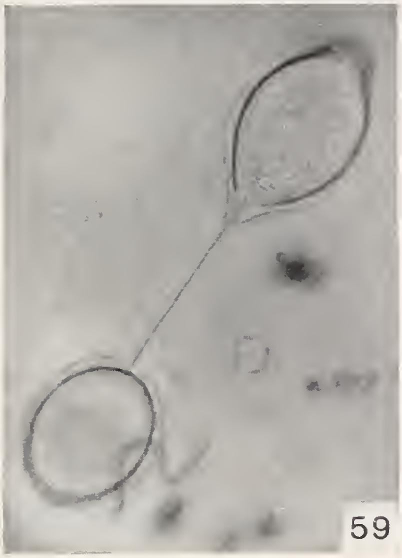
Parthenogenetic Merogones

The non-nucleate half of the *Sphaerechinus* egg, although larger in proportion than that of the other eggs studied and containing both mitochondria and yolk granules, does not develop as well. Fertilization membranes well separated from the surface, as is characteristic of the normal egg, are thrown off, often while still in the hypertonic solution. The egg then becomes amœboid, and fragments irregularly into a number of pieces. The irregularity of this process may be seen from Photographs 52–55 which are consecutive pictures of the same egg.

Haplones

The normal egg and the centrifuged whole egg after being subjected to parthenogenetic agents throw off fertilization membranes but do not go far in development. The centrifuged whole egg usually becomes

PLATE VI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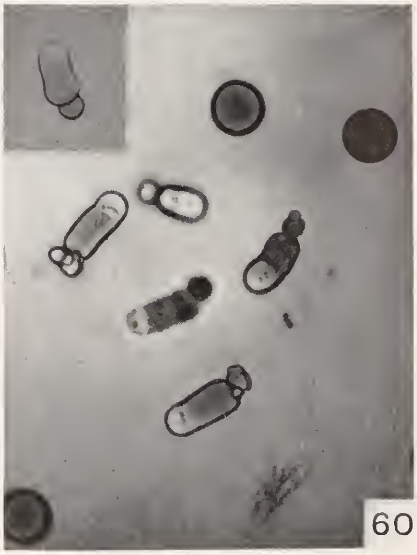
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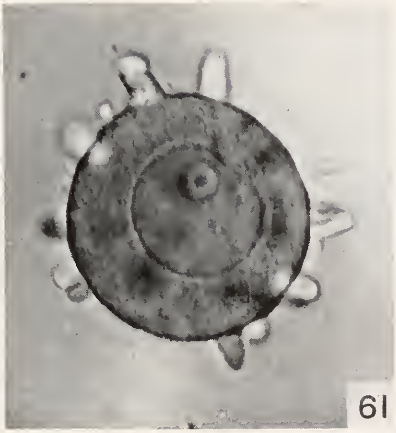
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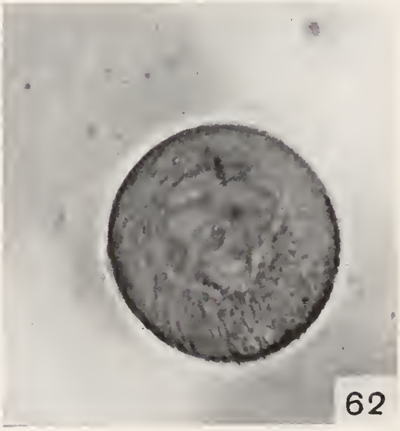
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Photographs 59–62

Immature eggs of *Parechinus microtuberculatus*.

Photograph 59. Centrifuged egg, almost broken apart. Note thin layer of material over both parts and over connecting strand.

Photograph 60. Change of shape and breaking up of normal immature egg, one hour after hypertonic sea water.

Photograph 61. Normal immature egg in sea water showing protrusions wherever sperm have hit the surface.

Photograph 62. Immature egg after hypertonic sea water and return to sea water with sperm; no protrusions on the surface.

amœboid and breaks into a number of fragments just as the non-nucleate fraction does (Photograph 56).

A peculiar phenomenon was observed in the centrifuged whole eggs and the white (nucleate) halves kept for a long time (one hour) in the hypertonic solution. The nucleus became elliptical, the long axis of the ellipse always parallel with the planes of stratification. As it later moved down through the clear layer it moved with its long axis in any direction. The ellipse almost immediately rounded out into a sphere when transferred to sea water. The cause and meaning of the ellipse is not known. An elliptical nucleus has been observed also in the centrifuged eggs of *Arbacia punctulata* after fertilization, but here it seems to occur at a definite stage in development, just prior to its breaking down (See Fig. 28 of my 1932 paper).

A cleavage plane in the parthenogenetic centrifuged egg frequently comes in across the short axis of the egg (but not usually at its narrowest part) while the nucleus is still intact (Photographs 57, 58) just as in *Parechinus*, showing again that a cleavage plane may be independent of nuclear changes.

The fertilized merogones cleave and develop quite normally, as previously described (1933).

Immature Eggs

Since the immature egg differs from the mature in having a large amount of material in the germinal vesicle, which mixes with the egg substance on maturation, it would be of great interest to get the non-nucleate portion of an immature egg to develop. This has been tried, and although not successful, some interesting phenomena have been observed.

(1) Immature eggs incompletely broken apart by centrifugal force were put in a hypertonic solution. While still in the solution, a thin layer formed on the outside of both the nucleate and non-nucleate parts and also on the connecting strand (Photograph 59). No fertilization membrane was formed, but the layer of material resembled the ectoplasmic layer characteristic of mature eggs activated either by fertilization or by parthenogenetic agents.

(2) When normal uncentrifuged immature eggs were treated with a hypertonic solution, they became elongate, with the germinal vesicle at one end; the other end pinched off a small piece. Subsequently this lower piece divided into two and later into many parts (Photograph 60). The same result was obtained with centrifuged immature eggs. Though this is not a typical cleavage, the process is similar and indicates some sort of change in the surface similar to that occurring during a true cleavage of a mature egg.

(3) The fact that the surface of immature eggs is definitely changed by the parthenogenetic agent is shown by their reaction to sperm. The normal immature egg forms characteristic protrusions at the surface wherever the sperm hit (Photograph 61); these resemble enlarged fertilization cones. Immature eggs which have been in hypertonic sea water and are then put back into normal sea water and sperm added, do not form these protrusions or react in any way to the sperm (Photograph 62).

Discussion

Although a further investigation of parthenogenetic merogony in four other species of sea urchin has not resulted in obtaining further development after the blastula stage, it has shown that the possibility of activating non-nucleate parts of eggs is fairly general. It may be that eggs will go only as far as the blastula stage without nuclei, and that nuclear material is necessary for differentiation, perhaps furnishing both chemical substances and genes.

All the non-nucleate parts will throw off a membrane, which is characteristic of the normal fertilized egg of its species, and they will all form an ectoplasmic layer and asters. The cleavage and early development does not seem to be dependent on any particular type of granule, for any type,—yolk, pigment or mitochondria, may be lacking in the different species and the egg still cleave. Cleavage is not dependent on a nucleus, for it may take place when it is completely absent or when it is present but intact and apparently inert. Nor is it dependent upon the presence of a spindle and asters as these may be centrifuged to another part of the cell and the cleavage plane come in without relation to them, as shown in a previous paper (1935). Cleavage seems to be dependent rather on the physical and chemical state of the protoplasm itself and of its surface layer. An orderly succession of cleavages is not necessary for the production of a many-celled organism. The cytoplasm can break up irregularly into fragments both when nuclear material is present and when it is not, and yet give rise to a blastula quite similar to that obtained by successive cleavages. The lability of cleavage planes and their disappearance and reappearance is characteristic of some of these non-nucleate fractions, just as was found for normal eggs deprived of oxygen (E. B. Harvey, 1927). Cleavage, budding and fragmentation seem to be different expressions of the same state of cytoplasm or of the cell surface, or both, in its effort to subdivide itself into smaller entities. Whatever the cell with a nucleus has been observed to do in this respect, the same cell without a nucleus has been observed to do, at least for a certain period. At the time of cleavage, a tendency for the egg to become amœboid seems to be an expression of the physical state of the surface, just as the appearance of asters is usually associated with the state of the interior protoplasm at this time.

SUMMARY

1. The stratification, by centrifugal force, of the eggs of the four species of sea urchin occurring at Naples is described.

2. The non-nucleate parts of the eggs of all four species can be activated by parthenogenetic agents. They throw off fertilization membranes characteristic of the species, and begin to develop.

3. Development of the parthenogenetic merogones of *Arbacia pustulosa* is similar to that of *Arbacia punctulata*.

4. The parthenogenetic merogones of *Parechinus* and *Paracentrotus* develop by a process of progressive fragmentation inside the fertilization membrane, similar to that occurring in the parthenogenetic normal egg which leads to normal free-swimming blastulæ.

5. Cleavage may take place in whole eggs while the nucleus is still intact.

6. Certain changes take place in the immature egg similar to those of the mature egg when treated with parthenogenetic agents.

7. No further development of eggs without nuclei has yet been obtained beyond the blastula.

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AN EXPERIMENTAL STUDY ON MITOSIS IN THE SOMATIC CELLS OF WHEAT[1]

H. W. BEAMS AND R. L. KING

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In the normal mitotic division of a cell the fundamental processes are seen as constituting a perfectly integrated whole. However, occasionally in nature and frequently under experimental or diseased conditions, these basic coördinated processes which occur in both the nucleus and cytoplasm become dissociated or suppressed, resulting in a failure to divide or abnormal division of the cell.

There are numerous agencies which have been reported as modifying or inhibiting cell division, namely: cold, heat, anæsthetics, basic dye-stuffs, injury, disease, poisons, hypertonic and hypotonic solutions, centrifuging, various types of radiation and hybridization. Recently, geneticists have made use of these methods in an effort to induce polyploidy in both normal and hybrid species of plants with the hope of producing more profitable economic varieties.

Our interest in this problem rests primarily in its cytological aspects, that is, to show how colchicine in certain concentrations and centrifuging at high speeds may affect the normal mitotic mechanism in rapidly dividing cells and, in addition, to analyze the mechanism that may eventually give rise to polyploids under experimental conditions.

Material and Methods

The variety of wheat which we have used in all experiments is known as "Red Durham." A large number of seeds were first soaked from 3 to 5 hours in tap water and from these only the most uniform in size and appearance were selected and placed in a moist chamber where they were allowed to germinate at 20° to 22° C. in the dark. After approximately 16 to 20 hours the growing parts of the wheat embryo were observed bursting through the surface of the swollen seeds. Some of the germinating seeds were then placed in various concentrations of colchicine ranging from 1 : 250 to 1 : 2000 for 30 minutes to 12 hours. After treatment with colchicine they were washed in tap water and fixed immediately or allowed to stand in a moist chamber for various intervals before fixing.

Others of the germinating seeds were placed in the rotor of the air turbine ultracentrifuge (Beams, Weed, and Pickels, 1933) and centrifuged at various speeds giving forces from 15,000 to approximately 100,000 times gravity. The best procedure for our studies has been to centrifuge the seeds at 100,000 times gravity for 10 minutes, after which they were either fixed immediately or after they had recovered for varying lengths of time.

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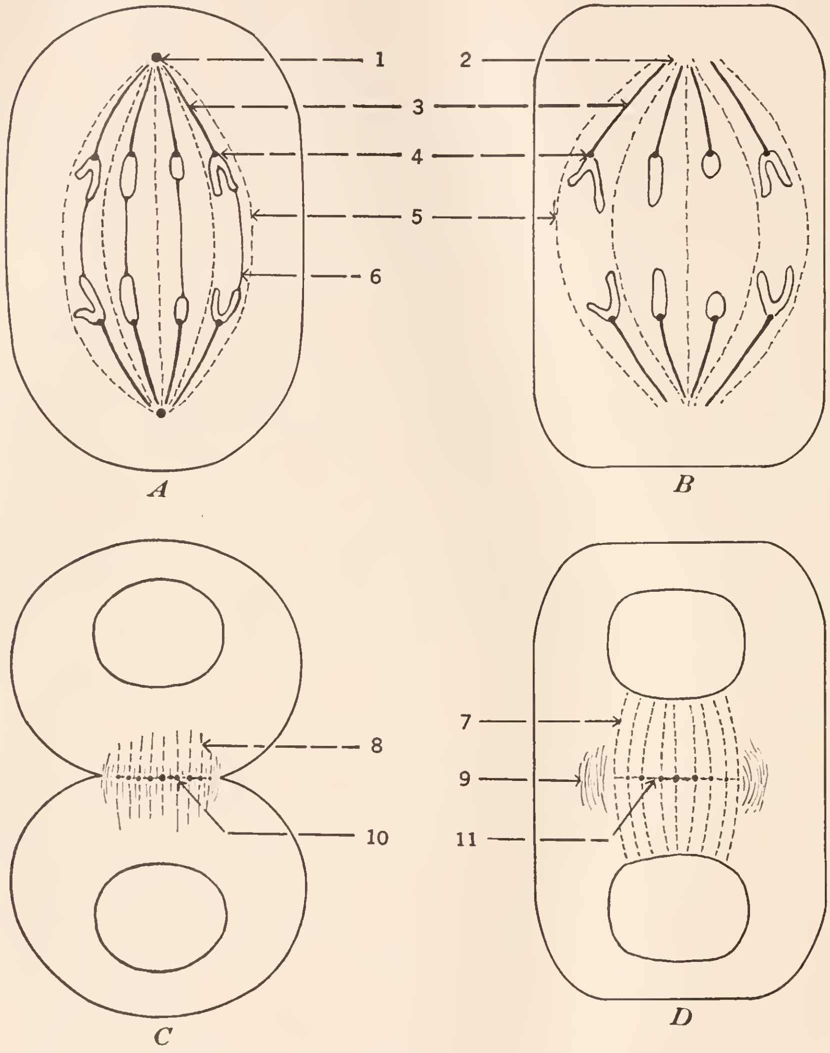
[1] Aided by grant from the Rockefeller Foundation for work on Cellular Biology.



TEXT FIG. 1. Diagrams showing the mitotic components in certain plant and animal cells. *A*, *C*, animal cells; *B*, *D*, plant cells. 1, centriole; 2, polar center; 3, traction, half-spindle, centromere, or chromosome fiber; 4, fiber attachment, centromere or kinetochore; 5, continuous spindle fiber; 6, interzonal fiber; 7, spindle of cytokinesis; 8, spindle remnant; 9, phragmoplast; 10, mid-bodies; 11, cell plate.

All material whether colchicine-treated or centrifuged has been fixed in Bouin's solution and subsequently stained in Heidenhain's hæmatoxylin.

Description

General

That a mitotic spindle can be differentiated from the surrounding cytoplasm during cell division in many animal and plant cells seems amply demonstrated. However, many questions concerning the origin, structure and function of the different elements of the spindle remain unanswered. Early studies upon the mitotic spindle and current elementary textbooks of biology generally describe it as composed of a single set of fibers. However, in animal cells it has been demonstrated to consist of a number of elements definitely correlated to function as a single unit (Text Fig. 1, *A–C*). In plants, too, it can be demonstrated that the spindle is made up of a number of different components derived from the nucleus, from the cytoplasm or from both (Text Fig. 1, *B–D*). No centrosomes or asters can be demonstrated in the higher plants comparable to those in many animal cells. However, physiological poles or centers where the spindle fibers converge seem to perform much the same function as do the centrosomes in animal cells. As far as we have been able to determine, no interzonal fibers comparable to those found in animal cells have been clearly shown for plants. Moreover, in the cells of higher plants the continuous fibers are usually described as giving rise to the "spindle of cytokinesis" and the phragmoplast which eventually forms the cell plate; in certain animal cells the continuous fibers appear to give rise to mid-bodies (see Fry, 1937, for a recent discussion on this point).

The Effects of Colchicine

The effects of colchicine upon dividing cells have been studied by a number of investigators (Dustin, 1934; Lits, 1934, 1936; Ludford, 1936; Brues and Cohen, 1936; Allen, Smith, and Gardner, 1937; Blakeslee and Avery, 1937; Havas, 1937; Brues and Jackson, 1937; Okkels, 1937; Richards, 1937; Nebel and Ruttle, 1938).[2] It seems to change the colloidal nature of the cytoplasm, thus inhibiting the physico-chemical changes that normally accompany cell division. In this way the achromatic spindle elements are prevented from forming, and the chromosomes are left stranded at the metaphase (Fig. 2). If, however, the cells are sufficiently affected by the colchicine, the divided chromosomes will break down and form a restitution nucleus with double the somatic number of chromosomes present. In other words, the nuclear changes characteristic of mitosis proceed more or

[2] See also, Walker, R. I., 1938. *Am. Jour Bot.*, **25**: 280.

less normally to metaphase, where chromosome movements are arrested much longer than usual. After an abnormally long delay at metaphase the chromosomes which are double separate, but only for a comparatively short distance because of the absence of spindle fibers, and an ameboid nucleus is finally formed (Figs. 3, 33). Under suitable exposure to colchicine the same cell may pass through several cycles of this type, giving rise to a doubling of the chromosomes at each cycle (Figs. 6, 7, 29, 30). At each division of the chromosomes the cell body enlarges, as well as the nucleus, until the roots and shoots of the embryos so treated have grown greatly in diameter, simply as a result of the enlargement of the cells without division. The cytoplasm of such cells is greatly vacuolated and the nucleus, in addition to containing an abundance of chromatin and, frequently, vacuoles also, possesses numerous nucleoli. We have counted as high as 75 nucleoli in the nucleus of a cell of this type. In fact, the appearances of such cells in many ways are strikingly similar to those of certain tumor cells (compare with the figures of Levine, 1931). Cells affected to this extent seldom recover.

Tissues that are exposed to suitable concentrations of colchicine for the proper time readily recover. For instance, those that have

EXPLANATION OF FIGURES

All drawings were made by Miss Bernette Bohen from colchicine-treated cells of the roots of germinated wheat seeds.

FIG. 2. Cell arrested at metaphase. The chromosomes which have divided but which have not separated are much more loosely arranged than those in control cells at the same stage.

FIG. 3. Restitution nucleus resulting from a condition as in Fig. 1. An incomplete cell wall is shown.

FIG. 4. Binucleate cell.

FIG. 5. Binucleate cell undergoing mitosis.

FIG. 6. A "resting" cell that has passed through two or more divisions of the nucleus only.

FIG. 7. Metaphase of a cell as in Fig. 6. Many more than the normal number of chromosomes are present.

FIG. 8. Late anaphase showing a tripolar spindle.

FIG. 9. Metaphase plate showing numerous constrictions of the chromosomes. Not all the chromosomes in the cell are shown.

FIG. 10. One form of abnormal separation of the chromosomes in a cell that had partially recovered from colchicine treatment.

FIG. 11. Three cells derived from a single tripolar spindle as in Fig. 8.

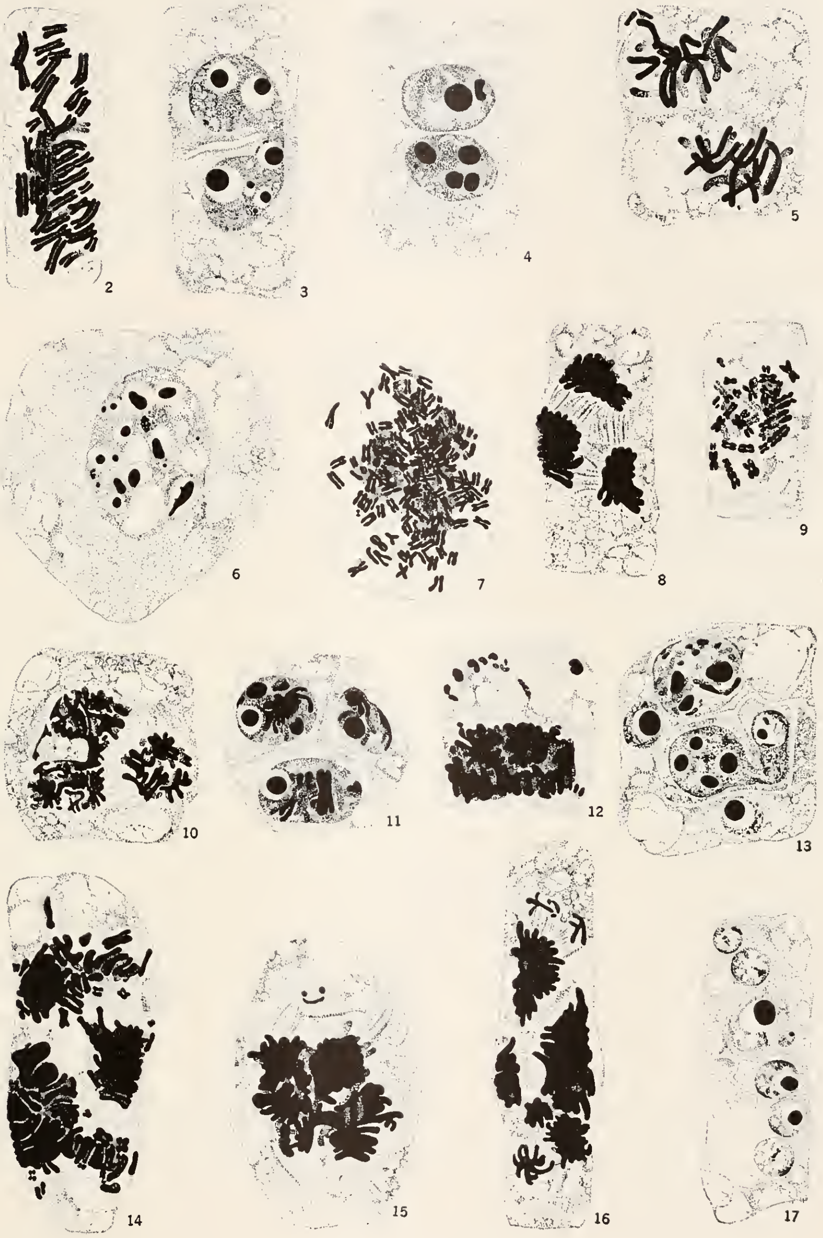
FIG. 12. Cell showing small spindles between the scattered individual chromosomes in a partially recovered cell.

FIG. 13. Three irregular cells. The large U-shaped peripheral one contains one large nucleus and two smaller ones. The central one contains an abnormally large bean-shaped nucleus, the other cell being a small non-nucleated one.

FIGS. 14, 15, and 16. Dividing cells with more than the normal number of somatic chromosomes. Various types of abnormalities that occur in the separation of the chromosomes are shown.

FIG. 17. One example of an abnormal cell that has resulted from an abnormal mitotic division similar to those illustrated in Figs. 14, 15, and 16.

PLATE I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passed through one mitotic cycle of the nucleus only may completely recover and, at the next mitosis, form a normal achromatic spindle mechanism and divide with double the somatic number of chromosomes. It is the production of this and similar conditions that the geneticists are striving to attain. If, on the other hand, the effect of the colchicine treatment has been such that the cell recovers after a long arrest of the chromosomes at metaphase, many types of mitotic abnormalities may arise. Because of the great number of chromosomes in our material it has not been possible to accurately count them. Nevertheless, with reasonable certainty it is possible to determine those cells that have formed some multiple of the normal diploid number.

In different regions of the same root in which mitosis has been arrested by colchicine we have found the chromosomes to vary from apparently the normal condition to that in which the chromosomes possess numerous constrictions (Fig. 9). Cells blocked at metaphase sometimes have their chromosomes abnormally scattered on the "equatorial plate" (Fig. 2). In fact, as pointed out by Brues and Cohen (1936), the chromosomes appear as though they actually repelled each other. Perhaps this condition is due to the absence of the controlling forces of the spindle fibers. When recovery takes place and the spindle or spindles start to appear, the loosely arranged chro-

EXPLANATION OF FIGURES

All figures were drawn by Miss Bernette Bohen from centrifuged cells of the roots of germinated wheat seeds. The centrifugal force in all cases has been directed approximately toward the bottom of the plate.

FIG. 18. Prophase, showing chromatin displaced to the centrifugal end of the nucleus.

FIG. 19. Metaphase, with chromosomes and spindle displaced to centrifugal end of the cell.

FIG. 20. An unusual case where the chromosomes have been thrown free of the metaphase spindle. Not all the chromosomes are shown.

FIG. 21. Late anaphase with most of the chromosomes separated from the spindle. The chromosomes are displaced centrifugally, the spindle centripetally.

FIGS. 22, 23, and 24. Cells in telophase, showing displacement of nuclei. No spindle fibers or phragmoplast were observed in these cells. They had evidently disintegrated.

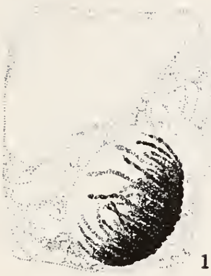
FIG. 25. Binucleate cell resulting from a condition like that in Figs. 21, 22, 23, and 24.

FIG. 26. A condition in our material where the chromosomes have been thrown centrifugally, and upon recovery have formed a restitution nucleus. Cytokinesis has taken place giving rise to one daughter nucleated cell (with a double set of somatic chromosomes present) and one daughter non-nucleated cell.

FIG. 27. Tripolar cell. Cells of this kind give rise to three cells with an unequal number of chromosomes.

FIG. 28. Cell showing constricted chromosomes that are shortened and thickened. This condition probably represents an injury effect. Not all the chromosomes in the cell are shown.

PLATE II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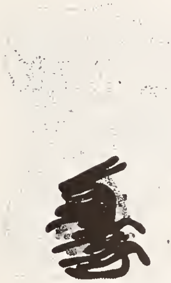
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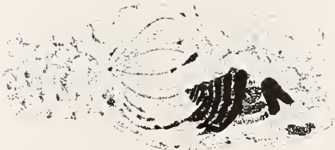
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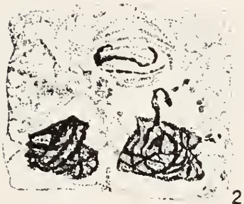
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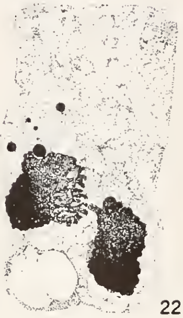
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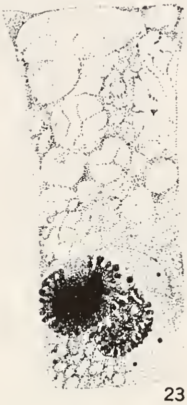
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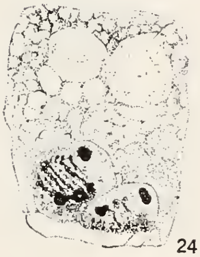
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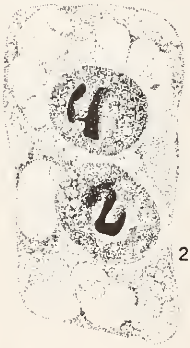
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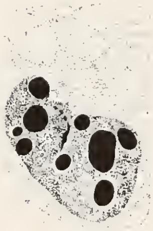
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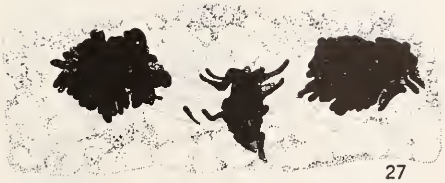
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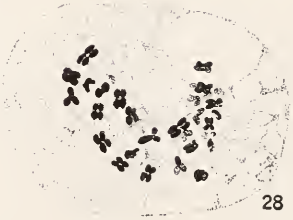
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mosomes have a tendency to segregate into groups, each of which apparently induces, or, at least, is associated with a definite spindle. The chromosomes in each group are often quite unequal in number (Figs. 12, 14, 15, 16, 32, 35, 37), thus giving rise to nuclei and cells of unequal size (Figs. 11, 13, 17, 31, 32, 34). If recovery has been incomplete and the spindles poorly developed, the chromosomes do not separate normally and no cell plate, or an incomplete one, is formed between the nuclei, depending apparently on the degree of recovery.

As previously mentioned, when no spindle is present the daughter chromosomes separate only a comparatively short distance and a restitution nucleus which is frequently greatly elongated or lobed is formed. If the chromosomes separate a greater distance during anaphase and telophase, generally a binucleate cell results, the nuclei often being of unequal size depending upon the equal or unequal partition of the chromosomes (Fig. 4). These cells may later form two metaphase plates (Figs. 5, 36). In cells like Figs. 3 and 33 an incomplete cell wall is formed. This condition has apparently resulted from a failure of the cell wall to cut through the large bean-shaped restitution nucleus which was formed before the cell wall was laid down. Many

Explanation of Figures

Photomicrographs of colchicine-treated and centrifuged cells from young roots of germinated wheat seeds. Figures 29 to 39 inclusive, colchicine-treated; Figs. 40 to 47 inclusive, centrifuged.

Fig. 29. Cell that had passed through two or more mitotic cycles of the nucleus only. Large ameboid nucleus containing many nucleoli is shown.

Fig. 30. Division of a cell like that shown in Fig. 7. Many more than the normal number of chromosomes is present.

Fig. 31. Five cells formed from an irregular mitosis as shown in Fig. 17.

Fig. 32. Three cells in mitosis which have resulted from division of a single cell.

Fig. 33. Same cell as drawn in Fig. 3.

Fig. 34. Four cells of unequal size derived from a single abnormal mitosis as shown in Fig. 31.

Fig. 35. Cell dividing abnormally with three groups of chromosomes; same cell as Fig. 14.

Fig. 36. Two sets of metaphase chromosomes of unequal size in same cell.

Figs. 37, 38, and 39. Other forms of multipolar conditions.

Figs. 40 and 41. Centrifuged cells showing displacement of chromosomes in prophase (40) and metaphase (41).

Fig. 42. Late anaphase showing displacement of mitotic figure. Bits of fragmented chromatin are seen in the centripetal end of the cell.

Fig. 43. Telophase showing the displaced nuclei at the centrifugal end of the cell; the phragmoplast has not been displaced. Such a cell appears to give rise to a condition like that shown in Fig. 26.

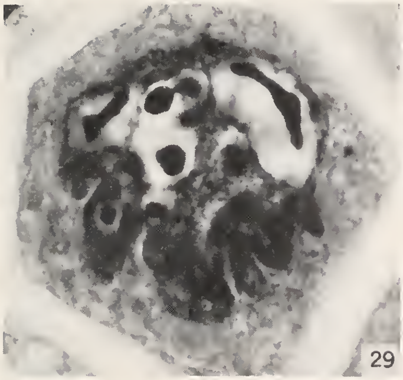
Fig. 44. Cell centrifuged at a slightly earlier stage in the mitotic cycle than that in Fig. 43. Here the phragmoplast is seen displaced to the centripetal end of the cell.

Fig. 45. Binucleate cell.

Fig. 46. Same cell as shown in Fig. 27.

Fig. 47. Another type of mitotic abnormality similar to that shown in Fig. 46.

PLATE III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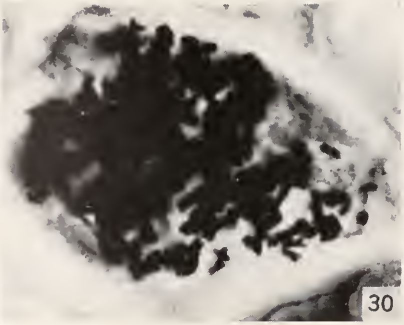
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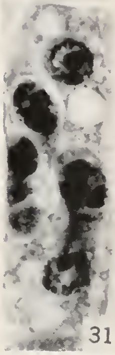
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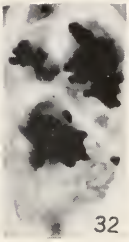
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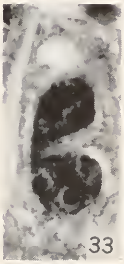
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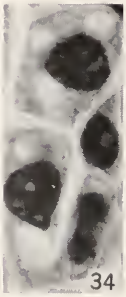
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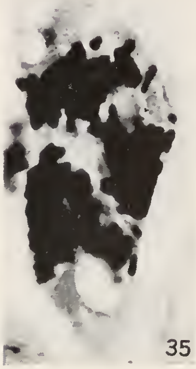
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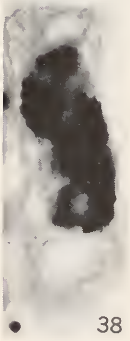
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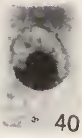
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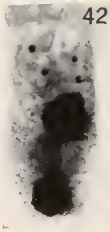
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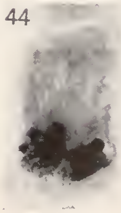
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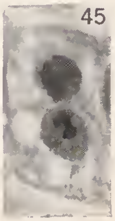
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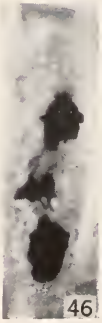
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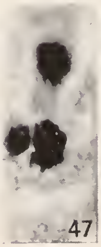
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such instances of this and similar conditions were observed in our material.

Abnormal mitoses like those illustrated in Figs. 8, 10, 12, 14, 15, 16, 32, 35, 37, 38, and 39 obviously give rise to cells with various chromosome numbers. It is highly probable that cells of this type included in a plant with normal or polyploid cells that have recovered from the colchicine treatment divide for only a short time and eventually disappear. However, we have not made a definite study of this point.

Effects of Ultracentrifuging

Plant cells and tissues have been centrifuged by several workers (Mottier, 1899; Andrews, 1915; Němec, 1915, 1929; Schaede, 1930; Kostoff, 1935, 1937, 1938; Luyet, 1935; Luyet and Ernst, 1934; Beams and King, 1935; etc.).

The general effect of strong centrifugal force (150,000–400,000 times gravity) upon the nucleus and cytoplasm of plant cells, i.e., the stratification of the materials in the order of their relative specific gravity, has been recently reported by us and need not be discussed here (Beams and King, 1935).

During prophase the chromosomes are displaced by ultracentrifuging and sometimes fragmented in the nucleus as illustrated in Figs. 18 and 40. In the metaphase many different effects of the ultracentrifuging are noted; for instance, in Figs. 19 and 41 the whole metaphase plate of chromosomes has been displaced to the centrifugal pole. The spindle is usually greatly distorted and in some cases seems to have disappeared completely. Just how the cell recovers from this condition is not altogether clear since we are unable to observe them in the living condition. However, in many instances at least, they apparently form a more or less normal anaphase and telophase except that the division takes place in the centrifugal end of the cell (Fig. 42). If this condition persists, it results in great discrepancy in size of the resulting daughter cells. In addition to displaced mitotic figures, many cells are seen with bits of fragmented chromatin resting free in the cytoplasm (Figs. 22, 42). In other cells which were fixed in anaphase following recovery from centrifugation in the metaphase multipolar spindles are present (Figs. 27, 46, 47). These spindles were probably formed as a result of the dispersal of the chromosomes by centrifuging. Conditions of this kind give rise to cells of unequal size and chromosome number. The chromosomes in relatively rare cells are shortened, thickened, and show various constrictions (Fig. 28), probably an indication that they have been injured as noted above. We have observed similar constrictions following colchicine treatment.

In a few cells which had been exposed to 100,000 times the force of gravity the chromosomes have been thrown free of the spindle (Fig. 20). Sometimes bits of chromatin which presumably contain the kinetochore or centromere remain attached to the spindle. This condition, which is relatively rare in wheat cells, is not uncommon in centrifuged embryonic chick cells (Beams and King, 1936). In anaphase and telophase stages also the chromosomes may be completely separated from the spindle (Fig. 21). In this cell the chromosomes have been thrown centrifugally while the spindle with a few bits of chromatin has been displaced centripetally. Here, then, as in other cells (Schrader, 1934; Luyet, 1935; Beams and King, 1935; and others), the chromosomes are heavier and the spindle substance is lighter than the surrounding cytoplasm.

In like manner, cells centrifuged in later telophase usually have their chromosomes or developing nuclei displaced to the centrifugal end, the "spindle of cytokinesis" and phragmoplast to the centripetal end of the cell (Figs. 22, 23, 24, 44). This condition almost invariably gives rise to a binucleate cell (Figs. 25, 45). Whether cytokinesis takes place in cells of this type apparently depends upon whether or not the phragmoplast had actually started to differentiate a cell plate. If the cell plate has started to appear in the center of the spindle, it is usually not greatly displaced (Fig. 43). Such a cell undoubtedly gives rise to a condition such as that shown in Fig. 26. Sometimes instead of the two displaced nuclei fusing as in Fig. 26, they remain distinct, thus forming one daughter cell with two nuclei, the other daughter cell being without a nucleus. If, however, the nuclei are displaced at a slightly earlier stage (Fig. 44), before the cell plate has definitely started to form, the "spindle of cytokinesis" is displaced to the centripetal end of the cell. In such cases no cell plate or wall is formed and a binucleate cell results much in the same way as that described by Němec (1929) in cells treated with nicotine. Both nuclei in cells of this kind form metaphase plates and divide simultaneously. If the spindles are situated close to each other, they apparently fuse in a manner suggested by Jørgensen (1928) but we have never actually observed this condition.

Discussion

There are numerous agents (see introduction) that will allow the cells to undergo karyokinesis without the usual accompanying cytokinesis, but none are more suitable for this purpose than the narcotic alkaloid colchicine. As pointed out by Ludford (1936), Dixon (1905) seems to have been the first to observe many cells in division after treatment with colchicine. He concluded that its effect was to "excite karyokinesis." However, Dustin (1934) and Lits (1934) were the first to suggest that the effect of the drug was that of a mitotic poison. More recently papers by Ludford (1936), Brues and Cohen (1936), Allen, Smith, and Gardner (1937), Blakeslee and Avery (1937), Havas (1937), Brues and Jackson (1937), Nebel and Ruttle (1938) and others have shown that colchicine specifically inhibits the formation of an achromatic spindle so that mitosis is blocked at metaphase. After a long delay at metaphase the chromosomes do not separate normally and cytokinesis does not take place, thus giving rise to a double number of chromosomes within the cell. We have been able to substantiate these general facts in this paper. In addition, the recovery stages of the cell from the colchicine treatment are interesting when viewed in the light of the mechanism of mitosis.

The fact that an incompact metaphase plate is formed in colchicine-treated cells without spindles indicates that the spindle fibers exert a definite force which bears upon the arrangement of the chromosomes at this stage. Also, when the chromosomes are loosely arranged at metaphase and allowed to recover, separate spindles appear in connection with the various groups of chromosomes, regardless of their size. Such conditions would seem to indicate that in this material the chromosomes are capable of organizing or inducing a spindle. (However, see the recent work of Gross, 1936, and Harvey, 1936.) It is a fact of interest that in colchicine-treated cells the arrested metaphase chromosomes are apparently incapable of forming the traction or centromere fibers which certain observers have described for the normal condition (Darlington, 1936; Carothers, 1936).

Richards (1937) has shown that the effects of colchicine on yeast are to stimulate growth, instead of arresting it, as is the case in wheat and in many other tissues. Unpublished observations indicate that the division rate of *Paramecium multimicronucleatum* is not decreased by colchicine in concentrations up to $\frac{1}{4}$ per cent. These observations are in agreement with the general view that the action of colchicine on mitosis is to inhibit the development of the achromatic spindle, since it is generally believed that yeast divides by amitosis.

It has long been held (see Sharp, 1934, for literature) that the cell plate in higher plants is formed directly from the "spindle of cytokinesis" and phragmoplast which are thought to be a product of the continuous fibers of the spindle, although this is denied by Robyns (1926). If this condition is true, then it is easy to understand why no cell plate or cell wall is formed in colchicine-treated material. Incomplete cell walls that sometimes form are, no doubt, in some way due to the abnormal development and function of the phragmoplast in partially recovered cells. When the chromatic (chromosomes) and achromatic (spindle and polar centers) elements are thrown out of step at metaphase by the late appearance of the achromatic elements the chromosomes usually proceed to form a restitution nucleus. The abnormally late appearance of the phragmoplast in relation to the chromosome cycle, while sometimes capable of giving rise to a cell plate and wall, is unable to cut through the restitution nucleus. It would seem, therefore, that the mechanism that produces cytokinesis is incapable of cutting through the nuclear substance.

It is now definitely known from the works of a number of authors (see Heilbrunn, 1928, for literature) that mitotic division is accompanied by definite changes in the viscosity of the cytoplasm, which are essential for spindle formation. Most of the methods that have been used to inhibit cell division or dissociate karyokinesis from cytokinesis seem to do so by preventing these normal viscosity changes. By centrifuging normal and colchicine-treated material at the same speed for the same time we have attempted to determine whether or not an effective dose of colchicine changes the viscosity of the cytoplasm. As a result we have found that the colchicine-treated cells show a more marked displacement of the chromosomes than does the control material, particularly at the metaphase stage. This indicates that a lowering of the viscosity in the colchicine-treated cells has taken place. However, it should be pointed out, as shown by Nebel and Ruttle (1938) for *Tradescantia* cells that cyclosis is not noticeably affected by the colchicine treatment. Colchicine in suitable concentrations to arrest mitosis in wheat cells does not prevent the normal cyclosis in *Paramecium*, nor the changes in viscosity associated with locomotion in Ameba.

In certain grain hybrids various observations suggestive of gonomery as described for certain animals (Wilson, 1925) have been published by Bleier (1930), who concludes that not only do species differ as to behavior of chromosomes, but also as to that of their spindle substance. This, he thinks is closely associated with the chromosomes—he even suggests that each chromosome may have individual spindle substance which is different from that of other chromosomes of the same species. Darlington and Thomas (1937), who have published on similar hybrids, believe that there are two spindles, one derived from the cytoplasm (spindle) and one from the fiber attachment of the chromosomes (centromere spindle). Normal behavior in mitosis involves the integration of these two, but either may appear independently. In hybrids the normal relationship of these is disrupted, with resulting abnormal divisions much as in Bleier's hybrids.

Various experimental treatments will cause similar abnormal divisions in somatic cells of non-hybrid material as shown by our observations.

Wasielewski (1903, 1904) reported that somatic cells treated with chloral hydrate divide amitotically. Němec (1904) repeated the experiments of Wasielewski and was able to confirm, in general, his observations but could not agree with his interpretation that chloral hydrate caused the cells to divide by amitosis. On the other hand, Němec showed that the treatment with chloral hydrate produced an arrested mitosis resulting in many types of mitotic abnormalities. Němec further suggested that the polyploid condition frequently resulting from treatment with chloral hydrate eventually disappeared from the tissue by a process of heterotypical reduction comparable to that occurring in spore mother cells. This latter assumption has not been generally confirmed. Kemp (1910) and Sakamura (1920) particularly have made extensive studies of the action of chloral hydrate upon dividing cells. In general they report that its effects on dividing cells is to induce mitotic abnormalities which are not unlike those described in this paper.

Van Regemorter (1926) attributes the failure of normal spindles to form in chloralized cells to a destruction of the cell's polarity; recovery from the effect of the reagent involving a return to the proper polarized condition of the spindle substance.

Numerous methods for the production of polyploidy in somatic cells have been suggested by plant breeders, of which the following may be listed: (For a review of the literature on this subject see Winkler, 1916; Jørgensen, 1928; Lindstrom and Koos, 1931; Bhatia, 1938.)

1. The failure or incomplete separation of the chromosomes at anaphase with the formation of a restitution nucleus with double the normal number of chromosomes present.
2. The formation of a binucleate cell, due to the failure of development of the cell wall between the two resulting nuclei or its later disintegration. Some authors describe the nuclei as subsequently fusing and others as remaining separate.
3. The fusion of two neighboring cells into one, usually the result of injury in grafting experiments or to disease.
4. The union of the two metaphase spindles of a binucleate cell to form one giant one ("endo-duplication").

It will be evident from our description that we have observed in colchicine-treated and in centrifuged material all the conditions mentioned in (1) and (2) as well as variations of them. We have never observed a fusion of neighboring cells after the cell plate had definitely formed. Neither have we observed a fusion of metaphase spindles of binucleate cells, although we may assume that it does occur. We have observed what appeared to be a fusion of two separately induced spindles of the loosely arranged metaphase chromosomes in cells that had been treated with colchicine.

It should be pointed out that the normal development of the spindle in higher plants seems to differ somewhat from that in animal cells. Some investigators believe, as pointed out by Lawson (1900) and Allen (1903), that the spindle in higher plants instead of being bipolar from the first and under the control of definite polar centers passes through a series of multipolar stages in the course of its development. Accordingly, in higher plants the looseness or incompactness of the spindle fibers, particularly at the polar centers, as compared to the condition in animal cells, may possibly offer an explanation as to why in the apparently normal tissue of plants more polyploid cells and irregular mitoses have been found than has been reported for animal cells.

As previously pointed out, both plant and animal cells have been centrifuged by several workers (see Kostoff, 1938, for a recent review of the literature). In general it has been shown that the cell materials stratify in the order of their relative specific gravities. In most cases the chromosomes, particularly during mitosis, are displaced to the centrifugal end of the cell. Kostoff (1938) has been able to induce alterations in somatic chromosome sets of various plants. After centrifuging in an ordinary laboratory centrifuge he found that various results, such as the failure of the chromosomes to separate, of the cell to divide, and injury to the spindle, as well as the displacement of single chromosomes, have given rise to monosomic, trisomic, polysomic, and various other forms of polyploid cells.

For the most part, our work confirms that of Kostoff (1938) and others on the general results of centrifuging dividing cells. In addition, certain effects of centrifuging on the "spindle of cytokinesis" and phragmoplast in relation to cell plate formation should be considered here.

As seen above, there is good evidence that the "spindle of cytokinesis" and phragmoplast give rise to the cell plate. According to Timberlake (1900) and others the cell plate is formed by a fusion of swellings occurring on the middle portion of the continuous spindle fibers. More recently it has been emphasized that the cell plate appears as a continuous film among the spindle fibers but not on them (Sharp, 1934, for references). However, the origin of this material which collects at the equator, and forms the cell plate is unknown but it has been claimed, according to Sharp (1934), by Dembouiski and

Ziegenspeck (1929), Damehl and Ziegenspeck (1929) to be derived from the nucleus.

Notwithstanding the fact that in some plant material the cell plate forms independently of the spindle fibers, there is every reason to believe that in the typical condition the "spindle of cytokinesis" induces cell plate formation. This is especially clear from the work of Jungers (1931) on the endosperm of Iris. This is multinucleate, and after the last nuclear division, secondary spindles arise on the flanks of the spindles connecting daughter nuclei. These secondary spindles connect the nuclei with neighboring nuclei. The phragmoplasts grow out from the connecting spindles and meet in the secondary spindles, thus bringing about the inclusion of single nuclei within cell walls. In our material, if the spindle fibers and phragmoplast are displaced early enough in the cycle, no cell membrane or wall appears and the displaced spindle and phragmoplast eventually disintegrate. Accordingly, we conclude that the material forming the cell plate has been displaced in the centrifuge toward the centripetal end of the cell, where it rarely, if ever, forms a wall. This is interesting in view of our recent work on the eggs of *Ascaris* (Beams and King, 1937) where the cleavage substance ("surface active material") is displaced by centrifuging, thus preventing cytokinesis although karyokinesis takes place more or less normally.

Conclusions

1. Colchicine markedly interferes with the dynamics of cell division. Dividing cells are arrested for an abnormally long time at metaphase, at which time the chromosomes become loosely arranged and sometimes segregate into groups. No spindle or polar center is formed so that the daughter chromosomes do not separate normally. However, eventually they form a single large restitution nucleus or occasionally two nuclei. In either case, no cell plate or wall is formed if the treatment has been sufficient, thus giving rise to double the somatic number of chromosomes within the cell. Some cells may pass through several cycles of this kind.

2. Cells that have partially recovered from the colchicine treatment, particularly at metaphase, show many other types of mitotic abnormalities. We have counted as high as 7 spindles arising from one incompact set of metaphase chromosomes. Separate spindles may be induced by the abnormal grouping of the chromosomes at metaphase, regardless of the size, of the single groups. Such abnormal spindles occurring in a single cell eventually give rise to several daughter cells, usually of different sizes.

3. Evidence is presented which indicates that the effect of the colchicine treatment is to lower the viscosity of the cytoplasm, thus inhibiting the normal viscosity changes that accompany mitosis. This in turn prevents the formation of the polar centers and achromatic spindle.

4. The use of colchicine is an excellent method to disengage the nuclear from the cytoplasmic processes in mitosis; a condition which would seem to indicate that they are independent in causation but related in effect.

5. Centrifuging at high speeds stratifies the cell materials in the order of their relative specific gravity. Cells may recover from such treatment.

6. In a few cells at metaphase the chromosomes are thrown free of the achromatic spindle. Usually, however, the whole spindle is displaced to the centrifugal end of cell. Recovery and subsequent division in such cells vary from the normal condition to that in which multipolar spindles are present. Such figures give rise to more than the normal number of daughter cells.

7. Cells centrifuged while in anaphase or telophase before the cell plate has begun to form, have the chromosomes or developing nuclei displaced to the centrifugal end of the cell; the "spindle of cytokinesis" or developing phragmoplast to the centripetal end of the cell, where it seems finally to disintegrate. This condition usually gives rise to a binucleate cell.

8. Evidence is presented that the cell plate-forming substance or organizer is displaced in much the same manner as is the cleavage substance in the centrifuged eggs of *Ascaris*, resulting, in both cases, in the failure of normal partition walls to form.

9. Polyploidy is more frequent in plants than in animals, partly because of the fact that the polar centers are not definitely localized in the former.

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Vol. LXXV, No. 2 October, 1938

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE ACTIVITIES OF VARIOUS SUBSTITUTED PHENOLS IN STIMULATING THE RESPIRATION OF SEA URCHIN EGGS[1]

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INTRODUCTION

Whenever a series of related compounds is found to be capable of producing a given physiological effect, there always appears the possibility of relating the activities of the various substances to their molecular structure. In examining such possible relations it is important to distinguish between two different measures of the activity of a substance. Where the effect increases continuously with increasing concentration of the substance, the activities may be represented by the concentration required to produce an effect of a definite magnitude. But if the magnitude of the effect passes through a maximum with increasing concentration of the agent, the activity may be defined by the concentration required to produce the optimum (or maximum) effect or it may be defined by the magnitude of the optimum effect. It is also essential, when more than one molecular species of a substance exists, to determine which is the active form. Without such information no real comparison of activities can be made, since with different substances the concentration of the active form will depend upon the equilibrium conditions existing between the various molecular species.

Due principally to the work of Clowes and Krahl (1936) a large number of substituted nitro- and halophenols are now known that are capable of increasing the respiratory rate of marine eggs. They also exhibit the highly interesting reversible block to cleavage first demonstrated by these investigators (1934) with 4,6 dinitro-o-cresol. The effect of these agents is of further interest in that the increased respiratory rate is not accompanied by abnormal development, as is the case with the various oxidation-reduction dyes that had been previously (Barron, 1929; Runnström, 1930) shown to stimulate the

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[1] This investigation was supported in part by a grant from the Penrose fund of the American Philosophical Society.

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respiration of marine eggs. These substances are then of interest to the embryologist since there exists the possibility of harnessing the extra energy that is liberated, and the ability to stop cleavage in a reversible manner should help us discover the factors that cause it to go.

The substituted phenols are all weak monobasic acids and the question of the active molecular species relates to whether it is the anion or the undissociated molecule that is effective. In a previous publication (1937) we have presented evidence for the view that it is the anion that is the active form inside the cell, but that penetration must be effected as the undissociated molecule. The evidence is based in part on the existence of a pH effect. At lower pH's it requires a lower concentration of a given substituted phenol to produce the maximum respiratory stimulation or reversible block to cleavage than it does at higher pH values of the solution. But the calculated concentration of undissociated molecules is very closely the same at the different pH's. Field et al. (1934) first demonstrated this pH effect of 2,4 dinitrophenol on yeast. He interpreted the result to mean that the substance is active as the undissociated molecule. However, when we compared a number of different nitro- and halophenols on the basis of the concentration of undissociated molecules present at maximum effective total concentration, enormous differences were found. It appeared for the extremes that a million molecules of one substance would be required to produce the same effect as one molecule of another if the undissociated molecule were the active form. On the other hand, when the concentration of the anion inside the cell was estimated, assuming that the substances penetrate freely in the undissociated form, and using the value for the internal pH determined by Chambers and Pollack (1927) and the Needhams (1926) a more reasonable result was obtained. The different compounds then gave values of the same order of magnitude.

The calculations of the concentrations of the undissociated molecules and of ions in the solution and inside the cell are made from the usual mass action equations. The concentration of undissociated molecules, $[HP]$, present in the solution may be expressed as a function of the hydrogen ion concentration, $[H^+]$, and the total concentration, $[\Sigma P]$, by the equation:

$$[HP] = \frac{[H^+][\Sigma P]}{[H^+] + K}, \tag{1}$$

where K is the constant for the acid dissociation of the particular substituted phenol and $[\Sigma P] = [HP] + [P^-]$. The concentration of anions, $[P^-{}_i]$, inside the cell is given simply by the equation:

$$[P^-_i] = \frac{[HP_i]\,K}{[H^+_i]} \tag{2}$$

in which the subscript, i, refers to values for the inside of the cell. There are several assumptions involved here. Firstly, the value of $[HP_i]$ is taken to be the same as $[HP]$ in the solution. This follows from the evidence that the substituted phenols penetrate freely in the undissociated form. Secondly, it is assumed that activity coefficients may be neglected. The error involved in this assumption is certainly very small in comparison with other sources of error in the data. It is assumed, thirdly, that $[H^+_i]$ is not changed by the addition of these substituted phenols. We shall discuss this point below.

Combining equations (1) and (2) we have:

$$[P^-_i] = \frac{[H^+_o][\Sigma P]\,K}{[H^+_i]([H^+_o] + K)}, \tag{3}$$

where the subscript, o, refers to the solution and the subscript, i, to the inside of the cell. In the cases where K is large with respect to $[H^+_o]$ then $[P^-_i]$ will vary directly with the hydrogen ion concentration of the solution and with total concentration of substituted phenol, namely:

$$[P^-_i] = \frac{[H^+_o][\Sigma P]}{[H^+_i]}. \tag{4}$$

Where K is small with respect to $[H^+_o]$ we have

$$[P^-_i] = \frac{[\Sigma P]\,K}{[H^+_i]}. \tag{5}$$

In the situation represented by equation (4), $[P^-_i]$ is independent of K. Therefore those compounds having relatively large values of K will give the same value of $[P^-_i]$ when $[\Sigma P]$ and $[H^+_o]$ are the same. $[HP]$ at the same time will vary inversely with K as may be seen from equation (1). In the situation represented by equation (5), $[P^-_i]$ is independent of $[H^+_o]$. Therefore those compounds with small values of K will show a direct proportionality between $[P^-_i]$ and K for the same $[\Sigma P]$. $[HP]$ at the same time will be practically equal to $[\Sigma P]$. In either situation, then, $[P^-]$ and $[HP]$ will vary in a different manner, so it will not matter whether we investigate substances with large or with small dissociation constants, provided that the different compounds have sufficiently different dissociation constants. In other words, we could not hope to determine the active form if all the members of the series had very closely the same dissociation constant.

If the dissociation constants for the different substituted phenols were all small or all large we would want to know their values very exactly. But since in our series there are compounds with large values as well as small values available, this is not so essential.

The experiments reported here are an extension of previous work (1937) and involve a comparison of the activities of various substituted nitro- and halophenols. We employ here the first criterion of activity referred to above; namely, the concentration required to produce a definite effect. Data on the magnitude of the maximum stimulation are also presented. In addition a new type of experiment is reported in which the internal pH is altered, and the effect on the optimum concentration determined. Also the buffer capacity of eggs is estimated.

Material and Methods

The eggs of the sea urchins, *Strongylocentrotus purpuratus* (at Corona del Mar, California) and *Arbacia punctulata* (at Woods Hole, Massachusetts), were used in these experiments. These two forms behaved very much alike with respect to the concentrations of the substituted phenols required for maximum respiratory stimulation and reversible cleavage block as well as the magnitude of stimulation.

The oxygen consumption measurements were made by means of the Barcroft-Warburg manometers using the conical type of vessel with side-arm (Anhang). The substituted phenols are added from the side-arm after a preliminary series of readings (lasting usually one hour) on the untreated eggs. The solutions were buffered by means of glycylglycine, which we have recently (1937) shown to be highly suitable for buffering in the neighborhood of pH 8.0. The concentration of glycylglycine was generally 0.015 molar in carbonate-free sea water, prepared as previously described. The pH's were determined by means of the glass electrode (Beckman pH meter), the values for the egg suspensions in the vessels at the end of a run being taken as well as that of the initial solutions.

Results

The nitro- and halophenols[2] listed in Table I were investigated. In all cases a series of at least six different concentrations were employed in order to determine the one giving maximum stimulation. From the theoretical considerations based on the assumption that the substances are active as the anion, $[P^-_i]$ inside the cell, it was possible to determine what total concentration to employ at a given pH of solution. Thus a fairly closely graded series of concentrations could be used, which, if the assumptions were correct, should include that at which maximum respiratory stimulation is obtained.

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[2] The dichlorophenols were generously supplied by Dr. John Ruhoff of the Mallinckrodt Chemical Works, and were subsequently purified and their melting points checked. The other compounds were obtained from the Eastman Kodak Company.

The criterion for maximum respiratory stimulation involves a point which has not been emphasized in previous work with substituted phenols. With increasing concentration the respiratory rate increases, but beyond a certain concentration the increase does not remain constant with time. There is, at these higher concentrations, an initial rise in respiratory rate lasting for a short period of time followed by a steady drop in rate, which may finally reach values below that of the controls. This can be seen in Fig. 1, in which the rates of oxygen consumption in different concentrations of p-nitrophenol are plotted against time. With concentrations up to 6.7 × 10^{-5} molar the rate rises with time as does the control (untreated eggs) rate during

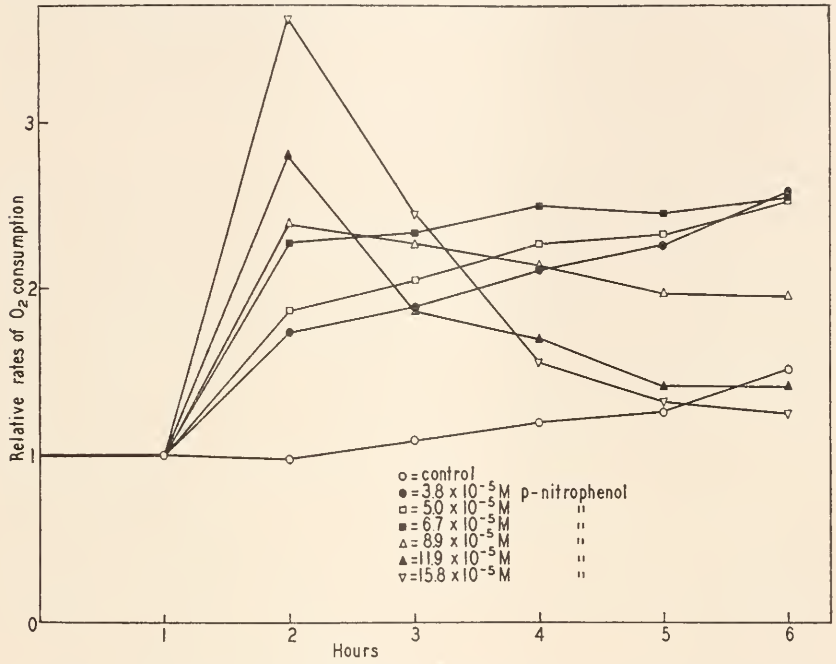

FIG. 1. Rates of oxygen consumption of eggs of *Strongylocentrotus purpuratus* in various concentrations of p-nitrophenol in glycylglycine-buffered sea water at pH 7.90. The reagent is tipped into the egg suspension at the end of the first hour. The relative rates of oxygen consumption are given on the basis of the oxygen consumption during the first hour as unity. Measurements begun at 43 minutes after fertilization. Temperature = 20° C.

this interval. But above this concentration there is an initial increase in rate followed by a steady drop, which in the case of the two highest concentrations, falls at the end of 6 hours to a value below that of the controls. This kind of variation of the oxygen consumption rates with time is found with the higher concentrations of all the substituted phenols that we have investigated. It is evident, with concentrations giving this initial rise followed by a decrease, that the particular value taken to represent the rate will depend upon the length of time after addition of the reagent during which the respiration is followed. One cannot, then, very well specify the amount of stimulation at these higher concentrations. We shall therefore take as the optimum concentration that concentration giving the greatest increase in rate of respiration without a subsequent decrease.

At the optimum concentration defined in this way we find that cleavage proceeds normally. The reversible cleavage block appears at concentrations just above this optimum; i.e., at concentrations at which there is an initial stimulation followed by a decrease. Thus in the experiment illustrated in Fig. 1, cleavage was blocked in the three highest concentrations of p-nitrophenol. Examination of the eggs from the vessels one hour after the end of the run gave the following percentages in the various stages:

	Number of Cells					Early blastulae
	1	2	4	8	16	
Control	1	1	0.5	0.5	0.5	96.5
3.8×10^{-5} M	1	1	0.5	0.5	0.5	96.5
5.0×10^{-5} M	1	1	0.5	0.5	0.5	96.5
6.7×10^{-5} M	1	1	0.5	0.5	0.5	96.5
8.9×10^{-5} M	1	1	0.5	30	64.5	3
11.9×10^{-5} M	1	39	60	0	0	0
15.8×10^{-5} M	1	89	10	0	0	0

The p-nitrophenol was added 15 minutes before the eggs were due to go into 4 cells. There is, as the data show, a time lag before cleavage is blocked which is particularly evident with the weakest blocking concentration. With higher concentrations the block is more immediate. At the same time the initial rise in respiratory rate is of much shorter duration in such solutions.

Using as the optimum concentration that which gives the maximum stimulation without a subsequent decrease with time, we may compare the various substituted phenols. In Table I these optimum concentrations, $[\Sigma P]$, at pH 8.0, are given for 15 substituted phenols. The amount of respiratory stimulation at the optimum is also listed. We shall not at this time attempt to compare the various compounds on the basis of magnitude of respiratory stimulation. The comparison on the basis of optimum concentrations must, as was previously pointed out, take into account the pH effect which showed that for a given substituted phenol, the same effect (e.g., optimum respiratory stimulation) was obtained at different pH's when there was the same calculated concentration of undissociated molecules present. The comparison of the different compounds is therefore first made on the basis of the concentration of undissociated molecules, $[HP]$, present in the solution at total concentrations giving optimum respiratory stimulation. These values are listed in the fourth column of the table, and it is evident that there are enormous differences between the different compounds. If the undissociated form were considered to be active (as the pH effect might indicate) then it would mean, for ex-

TABLE I

Concentrations required for optimum stimulation of rate of oxygen consumption in fertilized sea urchin eggs. Temperature = 20.0° C.

$[\Sigma P]$ = total concentration in solution at pH 8.0.
$[HP]$ = calculated concentration of undissociated molecules.
$[P^-_i]$ = calculated concentration of the anion inside the cell.

	$[\Sigma P]$	Max. resp. stimulation	pK	$[HP]$	$[P^-_i]$
	$M \times 10^5$	*per cent*		$M \times 10^{10}$	$M \times 10^6$
o-nitrophenol	373	37(?)	7.25†	2,780,000	62.2
m-nitrophenol	27.3	93	8.34†	1,870,000	3.4
p-nitrophenol	10.3	128	7.22†	146,000	3.5
2, 4-dinitrophenol	6.78	172	4.00†‡	67.8	2.7
2, 6-dinitrophenol	11.6*		3.59†	45.3	4.6
2, 4, 6-trinitrophenol	1090*	0	0.80‡	6.9	440
o-chlorophenol	47.2*		8.50§	3,590,000	4.5
m-chlorophenol	69.9	71	8.85§	6,130,000	3.5
p-chlorophenol	181	30	9.18§	17,000,000	4.5
2, 4-dichlorophenol	23.5	131	7.75§	876,000	6.2
2, 5-dichlorophenol	12.3	73	7.35§	224,000	3.6
2, 6-dichlorophenol	991*	0	6.80§	5,880,000	371
3, 5-dichlorophenol	20.8	39	7.92§	943,000	4.5
2, 4, 6-trichlorophenol	6.9*		6.00†	6,870	2.7
2, 6-dichloro-4-nitrophenol	4.87	140	3.70‖	24.4	2.0

* Value determined from cleavage block data, i.e., concentration just below blocking.
† From the International Critical Tables.
‡ From Scudder (see bibliography).
§ From Murray and Gordon (see bibliography).
‖ From Bader (see bibliography).

ample, that 700,000 molecules of p-chlorophenol are required to do the same job as one molecule of 2,6-dichloro-4-nitrophenol. Since this appeared extremely unlikely, a comparison of the various compounds was made on the basis of the calculated concentrations of anions, $[P^-_i]$, inside the cell at total concentrations giving optimum respiratory stimulation. The assumptions involved in this calculation are given in the introduction. These values are listed in the last column of Table I and it may be seen that with the exception of three compounds the values are all of the same order of magnitude. Two of the exceptions; namely, 2,4,6-trinitrophenol and 2,6-dichlorophenol give no respiratory stimulation, the $[P^-_i]$ values being obtained from cleavage block data. The other exception, o-nitrophenol, gave a small stimulation of respiratory rate. But our sample of this compound was probably impure as judged by its melting point. Dr. Clowes has suggested that the impurity may well be p-nitrophenol, which would account for the slight respiratory stimulation.

The three exceptions may therefore be omitted since they are not respiratory stimulants. The same may possibly hold for o-chlorophenol which was not sufficiently investigated to determine whether or not it will stimulate the respiratory rate. The narrow range in which the values of $[P^-_i]$ lie contrasts strikingly with the great divergence in $[HP]$ values of the various substituted phenols. Of the two alternatives, the anions $]P^-_i]$ or the undissociated molecules $[HP]$, the evidence shows that it is the former that must be considered the active form inside the cell.

The calculated $[P^-_i]$ values for different substituted phenols vary, the extremes showing a threefold variation. The data, however, are not sufficiently accurate for the differences to be considered significant. There are various sources of errors in the data. The errors in the manometric method are probably negligible. Those involved in the pH determinations and in slight variations in the course of a run are negligible for compounds with high pK values but not for compounds with low pK values. The pK values which are taken from the literature (see superscripts in Table I) are themselves subject to some error. This, as was shown in the introduction, affects principally the $[P^-_i]$ values for those compounds with high pK. In addition, certain of these compounds, particularly the mono- and dichlorophenols, are rather volatile, so that during the course of a run a certain amount of distillation into the alkali well of the vessel occurs. The magnitude of this factor is difficult to assess. It would, of course, tend to make the calculated $[P^-_i]$ values too high.

EFFECT OF CHANGING THE INTERNAL pH

If the anion is the active form within the cell, then by increasing the internal pH (keeping the external pH constant) a lower total concentration of the substituted phenol should be required to give the optimum respiratory stimulation or the reversible block to cleavage. It is possible to increase the internal pH by the addition of a weak penetrating base such as ammonia. The pH change is readily demonstrated by first staining the eggs with neutral red, and, after washing, immersing them in the ammonia solution. The extent to which the red color fades (neutral red changes from red to yellow between pH 6.8 and pH 8.0) gives the increase in internal pH that has occurred.

The effect of increasing the internal pH on the concentration of dinitrophenol required for reversible cleavage block was investigated with eggs of *Urechis*. The solutions were prepared from carbonate-free sea water using the glycylglycine buffer and adjusting the pH with ammonia. In the control solutions NaOH was used instead of ammonia. It was found in the first place, that when sufficient ammonia was present to effect an increase in internal pH, cleavage was interfered with or proceeded abnormally. These same solutions when used on unfertilized eggs gave artificial parthenogenesis. With solutions weak enough in ammonia to have no effect on cleavage and no activating effect on unfertilized eggs there was no observable effect on the internal pH. We were unable to prepare an ammonia solution which would give an observable increase in internal pH without at the same time affecting cleavage or activating unfertilized eggs.

TABLE II

Cleavage inhibition of Urechis eggs with 2, 4-dinitrophenol in ammoniacal sea water. Experimental and control solutions both prepared from carbonate-free sea water and buffered at pH 8.43 with glycylglycine.

Concentration of 2, 4-DNP	Cleavage in ammonia solution	Cleavage in control solution
(Molar)	(*per cent*)	(*per cent*)
0	99	99
2.6×10^{-4}	95	95
3.9×10^{-4}	0	95
6.0×10^{-4}	0	90

It was possible, however, to demonstrate, with the weakest ammonia solutions that give an observable increase in internal pH, an effect on the concentration of dinitrophenol required for cleavage block. The results of such an experiment are given in Table II. The

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percentage of eggs that divided in the ammonia solution is seen to be as high as in the control. The cleavage here was rather abnormal (in regard to size of blastomeres) and only abnormal bottom-swimming embryos were obtained as compared with 90 per cent top swimmers in the controls. It may be seen from the table that the concentration of dinitrophenol required to block cleavage is considerably less in the ammonia solution than in the control. This result conforms then to the expectation, on the basis of the anion being the active agent inside the cell. The fact that the ammonia solution employed did not give normal later development does not seriously interfere with the interpretation, since we would not expect a summation effect with dinitrophenol.

BUFFER CAPACITY OF EGGS

One of the assumptions on which the calculation of the concentration of anions inside the cell is based is that the internal pH of the cell does not change in the solutions used. Since the substituted phenols are weak acids and penetrate only in the undissociated form,[3] an acidification of the interior might be expected to occur. This would be independent of the pH of the external solution and the extent of acidification should depend upon the total concentration of the substance. The amount of decrease in internal pH that would be expected would also depend upon the buffer capacity of the egg and the dissociation constant of the particular substituted phenol. We have attempted to settle this point by staining eggs with indicators that have their color change in the region of pH 4 to 6, but have been unable to find any that penetrate readily.

In place of this, a determination of the buffer capacity of egg material was made. For this determination eggs of *Strongylocentrotus* were first frozen at − 72° C. for several hours and rapidly thawed to room temperature. This completely breaks up the eggs, resulting in a fine granular suspension which we shall refer to as egg-brei. An egg brei containing originally 2 parts of eggs to one part of sea water by volume showed a pH of 6.35. In order to allow the suspension to be more easily stirred during titration, it was diluted with an equal volume of distilled water, which resulted in a slight rise in pH to 6.41. The titration curve of this diluted egg brei is shown in Fig. 2 (curve *A*), and a titration curve of ordinary sea water is given for comparison (curve *B*). In the 10 cc. of egg brei that was titrated there was $1\frac{2}{3}$ cc. of sea water along with the $3\frac{1}{3}$ cc. of egg material. Comparison of curves *A* and *B* (the latter is for 5.0 cc. of sea water) shows that the correction for the sea water present is negligible. The buffer capacity of the egg brei is evidently quite high as the figure shows. To lower the pH from 6.4 to 5.4, 1.65 cc. of 0.1 normal acid are required. For the same buffering action a univalent acid with a pK of 6.4 would have to be present in an initial concentration of 0.04 molar, or considering the eggs alone, 0.12 molar.

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[3] Beck and Chambers (1937) have recently presented evidence to show that salts of weak fatty acids can under certain conditions penetrate marine eggs.

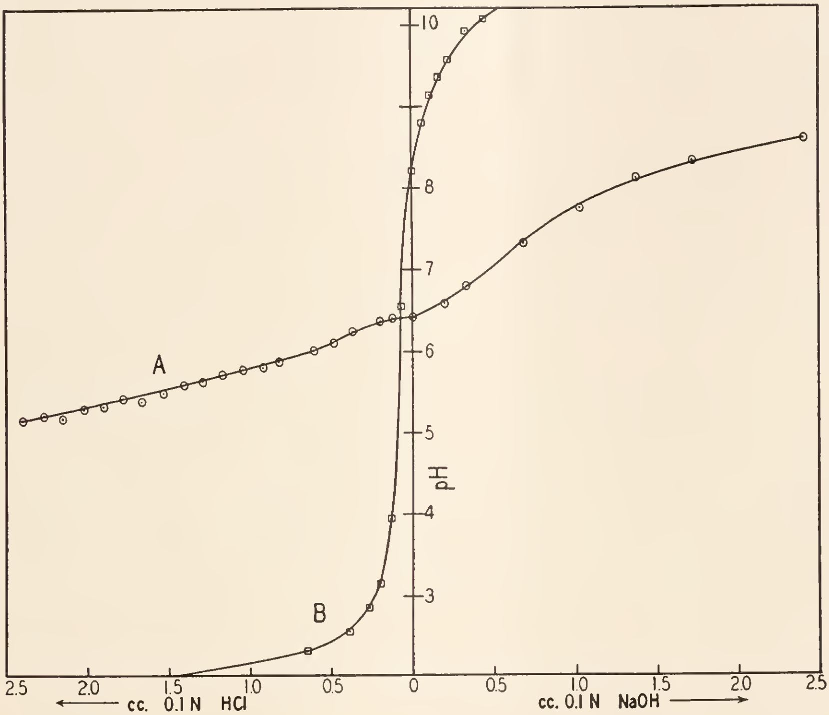

FIG. 2. Curve *A*; titration of 10 cc. of a suspension of sea-urchin egg brei (prepared by freezing at −72° C.) containing 3⅓ cc. of egg material, 1⅔ cc. of sea water, and 5 cc. of distilled water. Curve *B*; titration of 5 cc. of sea water.

We cannot, of course, assume that the buffer capacity determined in this manner gives a true picture of the situation in the living egg. Nevertheless, it would not be expected to be radically different. It is interesting to note that the egg brei shows a pH of 6.4 which is not very different from the value of 6.6 obtained by micro-injection of indicators (Needham and Needham, 1926).

For raising the internal pH with ammonia, at an external pH of 8, the total concentration of ammonia must be greater than 0.01 molar.

The optimum *total* concentrations of the different substituted phenols are all much lower than this.

It may be concluded, then, that the egg is sufficiently buffered to maintain a fairly constant pH in the presence of the concentrations of the substituted phenols employed in these experiments.

Discussion

In two recent articles, Krahl and Clowes (1938) present some tentative conclusions of their investigations on the action of substituted phenols. They are in agreement with our view (T. and H., 1937*b*) that penetration is effected in the undissociated form. Also, respiratory stimulation below the optimum is stated to depend upon the anion concentration inside the cell. But cleavage block and initial respiratory decrease (beyond the optimum), is considered to depend upon the concentration of undissociated molecules. The amount of stimulation at the optimum is then, according to these investigators, dependent upon the concentration of anions attained when the limiting concentration of molecules is reached, in the case of any given substituted phenol. The data obtained by Krahl and Clowes and by us agree, on the whole, very well. But in the experiments on the effect of changing the internal pH of the cell we differ. They find practically no effect on the total concentration required for cleavage block, whereas we find an effect in the direction expected according to our interpretation. Since the evidence from this source is partly responsible for the divergence in our respective views, a thorough investigation of the effect of changing the internal pH would be desirable. The difficulties involved in such experiments have been indicated above.

Field, Martin, and Field (1935, 1936) have compared the activity of several substituted phenols in stimulating yeast respiration. With those compounds for which dissociation constants were available, the calculated optimum concentrations of the undissociated form are given, since that is considered the active form by these authors. We have calculated from their data the concentration of anions that would be present inside the cell, using Gutstein's (1933) value of 6.2 for the pH of yeast. These values differ almost as much as do the figures for the optimum concentration of the undissociated form of the different substituted phenols that they list. It appears then that the situation in yeast is different from that in the marine eggs. It may be noted that the optimum concentrations of undissociated molecules obtained with yeast are much higher than with eggs, ranging from about 10 times as much in the case of the mono-nitrophenols to 1000 times as much with the dinitrophenols. It is not feasible at present to attempt to resolve the differences between yeast and marine eggs. It is evident, however, that the wide differences in $[HP]$ obtained with the various substituted phenols in yeast do not support the view that the undissociated form is active.

Recently Bodine and Boell (1938) have reported experiments on grasshopper embryos in which no pH effect is manifest with suboptimal concentrations of dinitrocresol. Actually their curves show a slight increase in respiratory stimulation as the pH is lowered, but the effect is much too small to be interpreted simply on the basis of the variation in concentration of undissociated molecules. Possibly in the grasshopper egg the changes in external pH produce proportionate changes in internal pH. This would, in the case of substituted phenols with relatively large dissociation constants (see page 211) tend to result in a constant or only slightly varying $[P^-_i]$ and would, according to our view, explain the absence of a pH effect on the respiratory stimulation. The more marked effect which they obtained with a stronger than optimum concentration might be similarly explained, especially since the stimulation drops off quite rapidly with concentrations above the optimum.

The differences in $[P^-_i]$ shown in Table I do not seem to us to be significant, for the reasons stated above. It appears likely, then, that the different substituted phenols have the same activity, considered on the basis of the $[P^-_i]$ required to produce the same effect. This is consistent with the view previously expressed by Clowes and Krahl (1936) that these compounds owe their activity to the phenolic OH group. The magnitude of the respiratory stimulation would depend then on the effect on the phenolate ion of the type of substitution in the benzene ring.

SUMMARY

1. Data on the concentrations required for optimum respiratory stimulation and reversible cleavage block in fertilized sea urchin eggs are presented for fifteen different nitro- and halophenols.

2. The experiments show that it is necessary to define the optimum as the highest respiratory increase that does not diminish with time.

3. Cleavage block occurs with concentrations just beyond the optimum defined in this manner.

4. Comparison of the calculated concentrations of undissociated molecules and of anions inside the cell for the different substituted phenols supports the previously expressed view that the anion is the active agent. Penetration is accomplished in the undissociated form, as previously shown.

5. The results of experiments in which the internal pH is raised also support this view.

6. Titration curves of egg brei indicate a high buffer capacity for the egg and support the assumption that the internal pH does not change in the presence of the substituted phenols.

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THE COPPER CONTENT AND COPPER TOLERANCE OF SOME SPECIES OF MOLLUSKS OF THE SOUTHERN CALIFORNIA COAST

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This report is mainly concerned with the results of analyses of specimens of some species of marine mollusks with reference to the variation of copper content with age or size, and the approximate upper limits of copper concentration in sea water that a few of them can tolerate.

Some quantitative determinations of the copper content of marine animals have previously been reported, particularly by Hiltner and Wichmann (1919), by Rose and Bodansky (1920), by Severy (1923), and by Galtsoff and Whipple (1931). These investigators found wide variations in the copper concentration of some of the species they studied, and Hiltner and Wichmann (1919) point out that both copper and zinc can be absorbed and retained in the tissues of oysters in much larger quantities than is needed for normal metabolism. Prytherch (1931) obtained evidence that copper is required for the attachment, metamorphosis, and survival of the oyster. Orton (1924) examined the English oyster for this element and mentions the poisonous effects on marine life of excess copper in sea water.

Copper occurs in combination as the chromoprotein hemocyanin in many species of arthropods and mollusks. For a review and bibliography of the work concerning this respiratory pigment, the reader is referred to the paper by Redfield (1934). Elvehjem (1935) has reviewed and discussed the biological significance of copper.

METHODS

The applicability of the sodium diethyldithiocarbamate method of Callan and Henderson (1929) to the determination of copper in biological materials has been established by McFarlane (1932) and by Tompsett (1934*a*, *b*, *c*, 1935). In this investigation, the procedure adopted was similar to theirs.

Water, redistilled in an all-Pyrex glass apparatus, was used for preparing reagents and rinsing containers. The reagents employed were tested and found copper-free. Tissues were ashed in silica vessels that had previously been treated with sodium acetate. The moist tissue was dried at 105–120°, weighed, and ashed in a muffle furnace at 380–420°. When necessary, small quantities of dilute nitric acid were added to hasten the oxidation of the carbon. A few grams of sodium phosphate were usually mixed with the dry tissue before ashing. The ash was treated with 6 N hydrochloric acid, or first with the 6 N acid, and then with redistilled water. An aliquot portion of solution was transferred to a glass-stoppered graduated cylinder which contained small quantities of sodium pyrophosphate and 25 per cent sodium citrate solutions. Both 4 per cent and saturated solutions of sodium pyrophosphate were used in the course of this work, and the volumes employed depended on the estimated quantities of iron present in the unknowns. The solutions in the cylinders were finally made alkaline to litmus with ammonium hydroxide. The standards contained 0.10 or 0.20 mg. of copper as cupric sulphate besides the other reagents.

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After cooling, 1 ml. or 2 ml. of a 2 per cent sodium diethyldithiocarbamate solution was added and the mixture shaken, and then 10 ml. or integral multiples thereof of benzene, toluene, xylene, or ligroin was introduced by pipette for extraction of the colored complex. The whole was then vigorously agitated. It was somewhat difficult to recover the solvent layer completely, or in the same quantities from standard and unknown. Therefore, the same fractional amounts were removed by pipette from each, and the colorimetric determination made. This procedure is correct if the quantities of organic solvent dissolved in the solution containing the standard and that to be tested are the same. Since n-amyl alcohol, which was used by McFarlane (1932), is fairly soluble in water, the other solvents which are much less soluble were substituted for the alcohol. Thus a possible error was avoided. Any water or precipitated material in the solvent layers was removed by centrifuging for a minute or two.

Since a few grains of sand were sometimes found present in the ash, even though considerable effort was made to remove them all from the moist tissues, these may have been a source of error. However, when small samples of sand, taken from the beach where many of the mollusks were obtained, were mixed with sugar charcoal and the above procedure carried out, the quantity of copper found was negligible. Although this is not evidence that such would always be the case, errors thus produced would be expected to be small.

According to Griffiths (1892), manganese is found in the respiratory pigment of the mollusk, *Pinna squamosa*. It is quite probable that this element occurs in other species of mollusks, and it was thus desirable to determine whether or not it could cause discrepancies in the analyses.

It was found that the reddish product formed when manganous chloride or sulphate react with sodium diethyldithiocarbamate is not readily extracted from the aqueous layer by benzene or toluene unless an electrolyte, such as, for example, HCl, NaCl, NH_4OH, is present. However, sodium pyrophosphate was found to form a non-ionizable compound with manganese as it does with iron when the solution is made basic with ammonium hydroxide. An alkaline solution may contain as much as 2.0 mg. of manganese and only .10 mg. of copper, but if sufficient pyrophosphate is present, no appreciable error results in the colorimetric determination for copper.

Zinc, which is also known to be present in mollusks, does not form a colored complex with the carbamate reagent. According to Callan and Henderson (1929), this element may be present in as high a concentration as 0.1 gram per 100 ml. and will not interfere with the copper determination provided sufficient ammonia is present and not too much of the colorimetric reagent has been introduced.

TABLE I

Copper recovery experiments

Series	Wt. of moist tissue	Quantity of copper added	Total copper found	Copper in tissue
	grams	*mg.*	*mg.*	*mg.*
A	30	0	0.158	0.158
	30	0.020	0.173	0.153
	30	0.060	0.211	0.151
B	30	0	0.071	0.071
	30	0.050	0.102	0.052
	30	0.080	0.139	0.059

COPPER RECOVERY EXPERIMENTS

Representative data obtained in a series of copper recovery experiments are given in Table I. In each series, the tissues of a number of mussels, *Mytilus californianus*, of various sizes were minced, drained fairly free of fluids, and well mixed. Portions were then weighed out. Copper was introduced by means of a standard cupric sulphate solution containing 0.10 mg. of copper per ml. In the second series of experiments, 3 grams of anhydrous sodium phosphate was added to each vessel. No sodium phosphate was used in the others, but good recoveries resulted.

TABLE II

The copper content of mollusks

Species	Length or weight of animals	Mg. of Cu per 100 grams of moist tissue	Mg. of Cu per 100 grams of dry tissue
Archidoris montereyensis	30.5 grams (one animal)*[a]	0.13	0.59
Astraea undosa (wavy top)		0.80	2.83
		1.26	4.34
Chione undatella (cockle)	2.7–3.9 cm., mean 3.6 cm. (7 animals) [b]	0.12	0.94
		0.20	1.24
Donax gouldii (bean clam)		0.06	0.41
		0.07	0.44
Helix aspersa (common terrestrial snail)	0.1–1.5 grams	1.63	10.7
	4.7–7.6 grams	10.8	56.1
Ischnochiton conspicuus (conspicuous chiton)	22–32.5 grams	0.37	1.03
		0.25	0.97
		0.28	1.08
Navanax inermis (striped mollusk)	51 grams (one animal) [c]	0.44	4.98
Mytilus edulis (bay mussel)		0.23	1.17
		0.37	2.38
Paphia staminea var. *laciniata* (rock cockle)		0.24	1.31
		0.18	1.48
	4.0 ± 0.2 cm.	0.21	1.68
	5.2 ± 0.1 cm. (three animals) [d]	0.18	1.44
Pecten circularis aequisulcatus (speckled scallop)	5.5–6.5 cm.[e]	0.31	2.40
Tegula gallina (speckled turban)		0.61	2.03
		0.61	2.30
Tegula viridula var. *ligulata* (banded turban)		0.81	3.25
		0.92	5.93
Tivela crassatelloides (clam)	[f]	0.21	1.19
		0.23	1.39

* Amount of copper per animal: [a] 0.039 mg.; [b] 0.006 mg.; [c] 0.213 mg.; [d] 0.009 mg.; [e] 0.043 mg.; [f] 0.013 mg.

RESULTS

For a description of the species investigated, refer to Johnson and Snook (1927). Some general data are given in Table II. In this instance, the tissues of from one to twenty animals were usually employed in an analysis without reference to the size of the organisms. Where ranges of dimensions are given, they represent the maximum for the shells. The best basis for comparison of results is that of the dry tissue, since it was generally not possible to remove the excess sea water completely from the moist tissues without endangering the loss of body fluids. Even heat-dried tissue may have caused inconsistencies if the animals had different proportional amounts of oxidizable lipoids, fats, and allied substances. This factor was believed to be less serious than those which would have to be reckoned with in the use of wet weights. A vacuum oven was not available. Concentration, in this

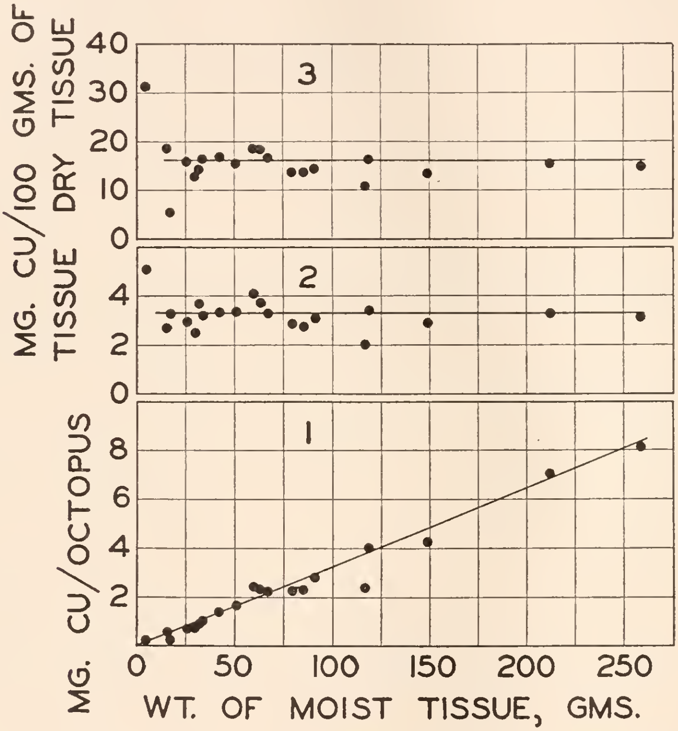

FIG. 1. Variation of copper content with size in the octopus, *Polypus bimaculatus*. Chart 1, mg. of copper per octopus; Chart 2, mg. of copper per 100 grams of moist tissue; Chart 3, mg. of copper per 100 grams of dry tissue.

case, means the calculated copper content per unit weight of tissue and does not imply a homogeneous distribution.

Severy (1923) found no copper in the Pacific coast clams, *Ensis americanus* and *Venus kennicottii*. However, the writer found copper in small concentrations in both *Donax gouldii* and *Tivela crassatelloides*.

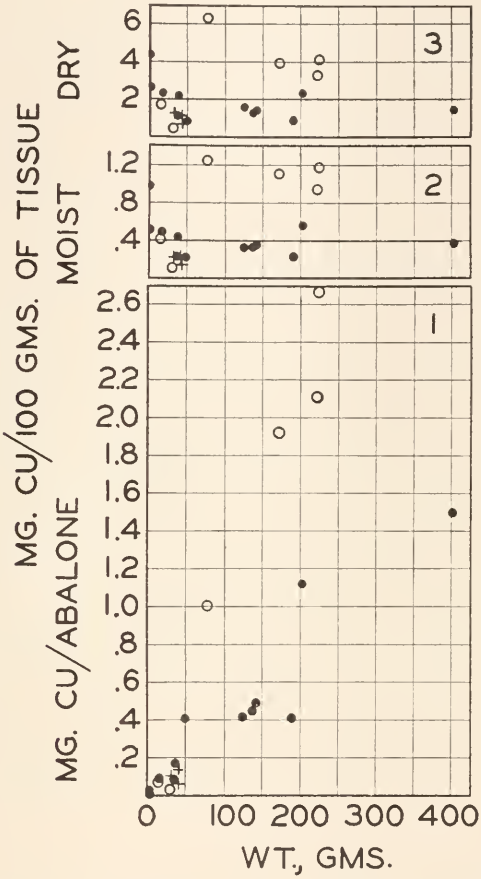

FIG. 2. Variation of copper content with size in the abalones, *Haliotis fulgens*, ○ = *H. cracherodii*, + = *H. rufescens*. The abscissa represents the weight of the moist tissue of each individual. Chart 1, mg. of copper per abalone; Chart 2, mg. of copper per 100 grams of moist tissue; Chart 3, mg. of copper per 100 grams of dry tissue.

It was of interest to determine the copper content of animals of different sizes of a few species and to learn whether or not there is any change in the copper content per unit weight of tissue with age or size. Data for six species are plotted in Figs. 1 to 6. Each plotted point represents the results of an analysis on a single individual with the exception of the data concerning the California sea mussel. External shells were excluded in the analyses.

The copper content in mg., s, of the octopus, *Polypus bimaculatus*, varies linearly with the weight in grams, m, of the animal (Fig. 1) and is given by the equation, $s = 0.031\,m$. Mean values of the copper concentration are 3.15 mg. of copper per 100 grams of moist tissue, and 15.6 mg. of copper per 100 grams of dry tissue. The data indicate that these concentrations are constant over the range investigated.

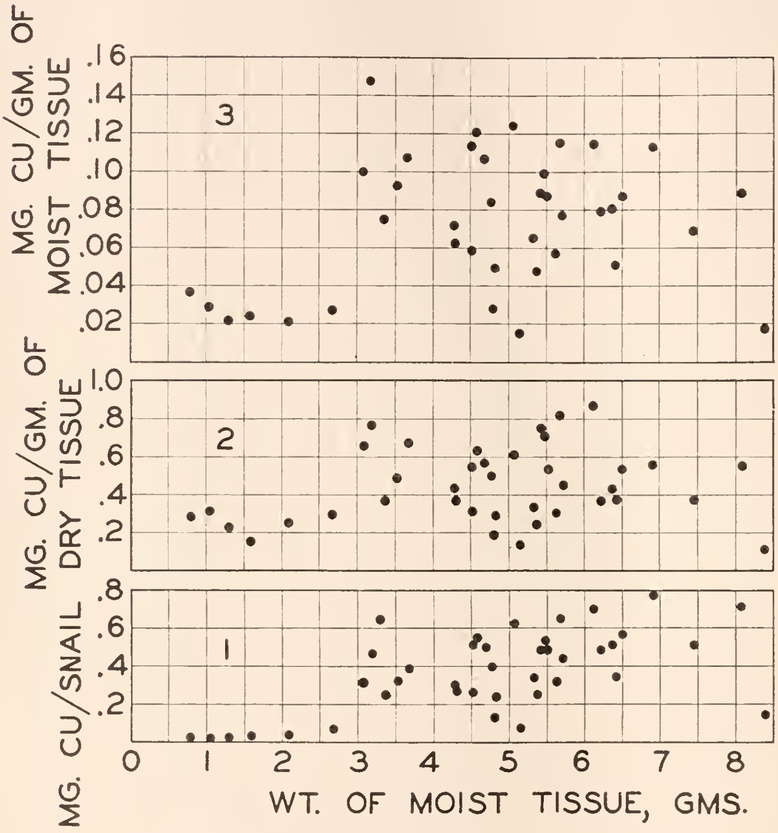

FIG. 3. Variation of copper content with size in the terrestrial snail, *Helix aspersa*. The abscissa represents the weight of the moist tissue of each individual. Chart 1, mg. of copper per snail; Chart 2, mg. of copper per gram of dry tissue; Chart 3, mg. of copper per gram of moist tissue.

Data concerning the species of abalones, *Haliotis fulgens*, *H. cracherodii*, and *H.* rufescens are plotted in Fig. 2.

Analyses were made of specimens of the terrestrial snail, *Helix aspersa*, for comparative purposes, and results are plotted in Fig. 3. These animals were collected in the late winter and early spring during a rainy period and, for the most part, were not in the resting stage at the time of collection. They were usually kept in the laboratory a few days prior to the analysis. The copper content of individuals which had approximately the same weight was found to be quite variable.

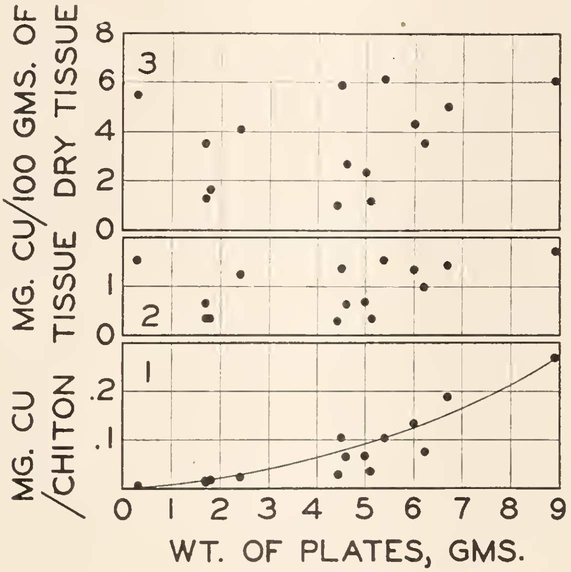

FIG. 4. Variation of copper content with size in the chiton, *Ischnochiton conspicuus*. The abscissa represents the weight of the plates of each individual. Chart 1, mg. of copper per chiton; Chart 2, mg. of copper per 100 grams of moist tissue; Chart 3, mg. of copper per 100 grams of dry tissue.

The copper concentration in both moist and dry tissues increases with increase in size of the animals. This fact is also shown by the data in Table II which were obtained by analyzing two groups of animals of a smaller and a larger definite weight-range. Severy (1923), likewise, found a much higher copper concentration in older specimens of the terrestrial mollusk, *Limax maximus*, than in less mature ones. Both the octopus and the snail contain sufficient copper to cause the ash of these animals to be quite blue.

Results of analyses for copper in the chiton, *Ischnochiton conspicuus*, and in the sea hare, *Tethys californica*, are given in Figs. 4 and 5, respectively. It is not known whether or not hemocyanin is the respiratory pigment in these species, whereas it is hemocyanin in the octopus and the snail, but in view of the relatively small amounts of copper in the sea hare it seems rather unlikely in this instance. Because of the difficulty of completely removing excess sea water from the chitons, the weight of the plates of each animal has been used as a measure of size.

The copper concentration in the dry tissue of the California sea

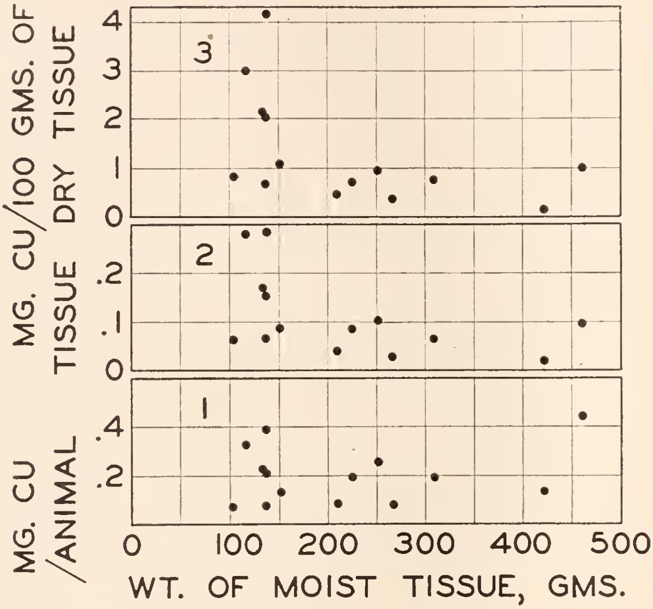

FIG. 5. Variation of copper content with size in the sea hare, *Tethys californica*. The abscissa represents the weight of each animal. Chart 1, mg. of copper per sea hare; Chart 2, mg. of copper per 100 grams of moist tissue; Chart 3, mg. of copper per 100 grams of dry tissue.

mussel falls off with increase in length (Fig. 6) of the valves. In general a number of animals were used in each analysis, and these were selected so that they had the same length within ±0.5 cm. of the mean for the larger sizes and ±0.2 cm. for the smaller sizes. Selection was made by using length of valves rather than the weight of the moist tissue because the procedure was then more rapid and more accurate. The weight of the moist tissue cannot be determined with the same accuracy as the length of the valves because of the accompanying sea water.

An investigation was made of the variation of copper content and concentration in different groups of organs obtained from young mussels and more mature ones, and data are given in Table III. The tissues of these animals were first hardened in a 2 per cent formaldehyde solution before removal from the valves. Since both the gonads and mantle contain eggs or sperm, these organs were grouped together. The foot is largely muscular tissue. No attempt was made to separate males from females. The decrease in copper concentration with increase in size is to be noted.

Chou and Adolf (1935) examined certain organs of five cadavers for copper and found a markedly higher concentration in the infants than in the adults. Sheldon and Ramage (1931) analyzed five foetal livers of man for copper and found a concentration 5 to 7 times that in adult organs. Perhaps copper plays a significant rôle in growth metabolism in many animals.

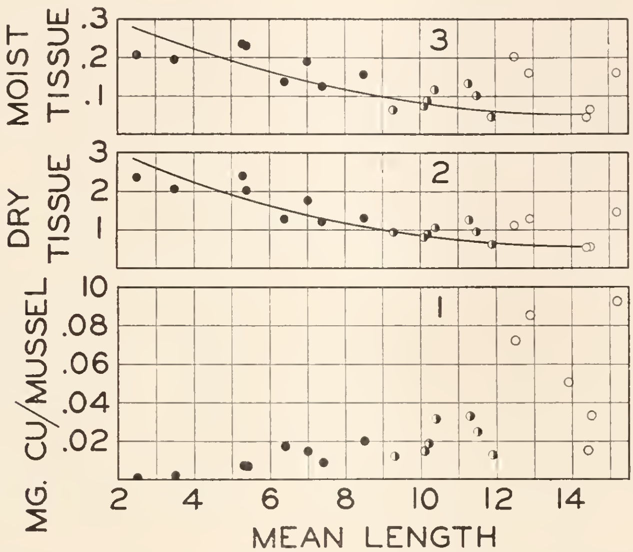

Fig. 6. Variation of copper content with size in the California sea mussel, *Mytilus californianus*. The abscissa represents the mean length of the valves when a number of animals were used. ○, one animal; ◑ , 2–3 animals; ●, 5 or more animals. Chart 1, mg. of copper per mussel (mean values); Chart 2, mg. of copper per 100 grams of dry tissue; Chart 3, mg. of copper per 100 grams of moist tissue.

Copper Content of Sea Water

In connection with the work concerning the copper tolerance of certain mollusks, it was desirable to know the copper content of the ocean water in which they live.

Using a procedure similar to that of Atkins (1933), determinations were made of the copper concentration in surface water taken 1,000 feet from shore at this station. The water was collected in a glass bucket and stored in Pyrex containers. Cursory examinations, made by adding 1 ml. of 2 per cent sodium diethyldithiocarbamate solution to 1 liter of sea water, extracting with 10 ml. of carbon tetrachloride or benzene and estimating colorimetrically, showed that the copper present could not be greater than 0.004 mg. per kg.

TABLE III

The copper content of groups of organs of the sea mussel, *Mytilus californianus* *

Length of mussels	No. of mussels used	Hepatopancreas, intestines and heart	Mantle and gonads	Muscles and foot	Gills and palps
cm.					
Range: 6.3–7.5	30	(*a*) 0.0038	0.0032	0.0022	0.0047
Mean: 6.9		(*b*) 27.4	23.0	15.8	33.8
		(*c*) 1.34	0.20	0.36	0.65
		(*d*) 9.35	2.52	2.26	7.70
Range: 6.6–7.7	30	(*a*) 0.0049	0.0034	0.0025	0.0052
Mean: 7.1		(*b*) 30.6	21.2	15.6	32.6
		(*c*) 1.66	0.20	0.36	0.55
		(*d*) 13.6	2.79	2.44	7.07
Range: 11.9–13.0	5	(*a*) 0.0159	0.0216	0.0042	0.0148
Mean: 12.5		(*b*) 28.2	38.2	7.4	26.2
		(*c*) 0.86	0.21	0.14	0.39
		(*d*) 8.70	3.26	1.00	5.44
Range: 11.8–12.4	4	(*a*) 0.0172	0.0234	0.0039	0.0102
Mean: 12.1		(*b*) 31.5	42.8	7.1	18.6
		(*c*) 0.85	0.23	0.12	0.27
		(*d*) 8.21	1.65	0.57	3.66

* The data for each group of organs of each series is placed in the following order:
(*a*) mg. of copper per group of organs per mussel (mean values),
(*b*) per cent of total copper present per mussel,
(*c*) mg. of copper per 100 grams of moist tissue,
(*d*) mg. of copper per 100 grams of dry tissue.

Twenty liters were electrolyzed, 1 liter at a time, between platinum electrodes at a potential difference of approximately 2 volts for at least 8 hours, and at a temperature of 70–90° during much of this period. The copper was dissolved from the cathode with dilute copper-free nitric acid, the solution was then neutralized with ammonium hydroxide, cooled, 1 ml. of the carbamate reagent introduced and the total extracted with benzene. A colorimetric determination showed

0.0010 mg. of copper present per kg. of sea water (salinity 33.68 per cent). An analysis of a 10-liter sample taken six weeks later showed 0.0014 mg. of copper present per kg. (salinity 33.63 per cent). There had been no rain in this region for several months.

COPPER TOLERANCE OF A FEW SPECIES OF MARINE MOLLUSKS

The approximate upper limits of copper concentration that some mollusks can tolerate, when kept in the laboratory under conditions to be described, were determined as follows.

TABLE IV

The copper tolerance of marine mollusks

Mg. of copper added per kg. of sea water		Time of survival, days				
		0	0.05	0.10	0.15	0.20
Species	Animals per jar					
Acmaea scabra var. *limatula*	4	16†		3†		3†
Fusinus kobelti	2–3	60		60		8†
Astraea undosa	2	30	14†	5†		5†
Haliotis fulgens	1–2	30	30	3†		
Ischnochiton conspicuus	2–3	60		60	10†	3†
Mytilus californianus	2	60		60	30	2†
Mytilus edulis	3	35		35		17†
Tegula gallina	3–4	60	60	15†		8†
Tegula viridula var. *ligulata*	4	60	60	25†		18†
Paphia staminea var. *laciniata*	3–4	100 alive at end of 30 days when 1.0 mg. of Cu added per kg. One animal alive at end of 65 days when 3.0 mg. of Cu added per kg.; another lived 58 days in latter concentration.				

† Dead at end of this time. All others alive at end of stated period.

In a series of jars, each of which contained 8 kg. of sea water, were placed a number of mollusks of a given species. Definite amounts of a standard cupric chloride solution containing 0.08 mg. of copper per ml. were then added to each jar. A continuous stream of air was passed in to keep the water well aerated, and distilled water added every day or two so that the volume and salinity were maintained constant. Many of the experiments were run in duplicate. Controls, to which no copper had been added, were run simultaneously. Temperature variations corresponded approximately to those of the sea. In general, there was a growth of algae, bacteria, etc. so that the animals were not without food. However, it should be pointed out that the natural habitat was not completely duplicated and other factors may have contributed to a decrease of the life span, such as the effect of added copper on the growth of certain organisms used for food.

Results are presented in Table IV. The upper limit of copper tolerance for most of the species investigated lies in a range of 0.10 to 0.20 mg. of added copper per kilogram of sea water and thus is about 100 to 200 times that normally present. However, the clam, *Paphia staminea* var. *laciniata*, can tolerate a much higher concentration. The addition of 3.0 mg. of copper as cupric chloride to a kilogram of sea water produces a noticeable cloudiness. Further introduction of copper alters the concentration of some of the other constituents because of precipitation.

Since younger mussels have a higher copper content per unit weight of tissue than more mature ones, it was thought that the former might tolerate 0.20 mg. of added copper per kilogram, but this was found to be untrue. The valves of the animals in this instance had a mean length of 5.3 cm.

SUMMARY

The copper content and concentration of several species of mollusks has been determined with particular reference to variation with age or size. In the snail, *Helix aspersa*, the amount of copper per unit weight of tissue increases with increase in size, whereas it decreases in the California sea mussel. In the octopus, *Polypus bimaculatus*, it remains constant. Samples of sea water of the Pacific were found to contain 0.001 mg. of copper per kilogram. The upper limits of copper tolerance of a few species of marine mollusks, when kept in the laboratory under given conditions, lies in the range from 0.10 to 0.20 mg. of added copper per kilogram of sea water. The clam, *Paphia staminea* var. *laciniata*, can tolerate a much higher concentration than the above.

I am indebted to many of the members of the staff of the Scripps Institution and to others for their helpful suggestions and kindly interest in this work and for their aid in the collection and identification of specimens.

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THE EFFECTS OF ULTRA-VIOLET RADIATIONS OF VARIOUS WAVE-LENGTHS UPON CLEAVAGE OF SEA URCHIN EGGS

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In a preceding paper retarding effects of radiations of λ2537A upon cleavage of eggs of the sea urchin, *Strongylocentrotus purpuratus*, were reported (Giese, 1938). The following account, extending the above study to wave-lengths of 2654, 2804, 3025, 3130 and 3660A, attempts to determine (1) the amount of retardation, if any, produced by known dosages of the above wave-lengths; (2) the amount of acceleration, if any, following low dosages; and (3) the region of the spectrum most effective in producing the effects noted.

The source of radiations (a water-cooled quartz mercury arc), the monochromator with optical parts of natural quartz, and the thermopile-galvanometer system for measuring intensities—were the same as those employed in the previous study. The data on intensities and the dosage ranges employed are summarized in Table I.

The animals were collected during the winter breeding seasons of 1937 and 1938 and the eggs were obtained and handled in the manner previously described. Approximately 300 eggs were used for each test. The experiments here reported were performed on fertilized eggs, irradiation beginning approximately 30 minutes after insemination. A dosage series of approximately 1024, 256, 64, 16 and 1 second was given at each of the wave-lengths. Three series of experiments were performed at each wave-length.

In most cases eggs from a single female were irradiated with a series of graded dosages of a given wave-length. In a few cases eggs from a single female were used for a series of dosages at each of two different wave-lengths. Thus in one instance eggs from one female were used for experiments with radiations of both λ2654 and λ2804A. The differences in effects of irradiation in this and other comparable cases were similar to those obtained with eggs of different females, the differences being of second order.

Following irradiation the eggs were pipetted into 1″ Syracuse watch-glasses, approximately 100 per dish, and were kept at 15 ± 1° C. Examinations were made at half-hour intervals, an attempt being made to obtain the time when a large proportion of the eggs had just completed a given cleavage. After the 64-celled stage had been attained observations were made every 12 hours. As in the previous study, so here, abnormal development of eggs was found to be only a small part of 1 per cent of the total number used. Controls always developed into plutei normal to all appearances, therefore the conditions were considered satisfactory for the investigation.

TABLE I

Intensity and dosage measurements

λ in A	Exper. Number	I in ergs/sec./mm.^{2}	Maximum dosage failing to retard cleavage in ergs/mm.^{2}	Total dosage range in ergs/mm.^{2}
2537	1	1.24	37.2	1.07–1585
	2	1.15	34.5	
	3	1.07	32.1	
2654	1	3.19	—	2.76–3444
	2	2.84	45.45	
	3	2.76	44.2	
2804	1	2.86	—	2.80–3090
	2	2.86	—	
	3	2.80	—	
3025	1	4.06	32.5	4.06–6840
	2	5.10	81.6	
	3	5.10	—	
3130	1	20.7	166	11.48–21,200
	2	11.48	—	
	3	13.74	210	
3660	1	38.5	(39,400)*	27.7 –74,500
	2	27.7	28,300	
	3	44.3	(74,500)*	

* At these dosages retardation was observed, but too slight to be accurately determined.

EXPERIMENTAL

In none of the experiments for any of the wave-lengths and dosages tried was there indication of definite stimulation and acceleration of the division rate, a finding in agreement with previous studies at λ2537A. The minimum dosages used are given in Table I. The eggs irradiated with a dosage below the retardation threshold cleave comparably to controls within the limits of accuracy of the investigation.

It is, of course, possible that the exact dose required for stimulation was not given or that even the lowest dosage was too great to induce acceleration.

While at the smaller dosages there was no retardation of the cleavage rate, such retardation was evident with sufficiently large dosages, the action of different wave-lengths being different only in degree. In each case retardation did not occur below a certain threshold (Fig. 2). In Table I are given data on the maximum dosage tried which failed to retard cleavage.

Retardation of cleavage was found to increase with dosage at all of the wave-lengths, as might be expected. After intermediate dosages of radiation, cleavage was normal and rhythmic, but after large dosages not only was cleavage delayed but it was often irregular; for example, whereas controls showed at most two cleavage stages at any given time, those irradiated with large dosages might show three or even four stages at one time. Occasionally abnormal cleavages appeared as well. Therefore it was often difficult to collect data for the larger dosages. None of the dosages used, however, were adequate to completely prevent cleavage.

Retardation following prolonged irradiation was apparent not only during cleavage but also at later stages in development. Thus at the time the blastulae of the controls were rotating within the fertilization membranes, those sufficiently irradiated were still immotile; when controls hatched from the membranes, those irradiated were still confined; when gastrulae formed, those irradiated were only beginning invagination.

In all cases eggs irradiated for 1, 16, and 64 seconds gave rise to blastulae, gastrulae and plutei normal to all appearances; in most cases those irradiated for 256 seconds did so as well. However, only in experiments at wave-lengths of 2537, 3130 and 3660A did plutei develop when eggs were given the maximum dosages recorded in Table I. In the experiments with large dosages at other wave-lengths the blastulae were as a rule abnormal and gastrulated abnormally. When plutei-like larvae developed they were abnormal, although in some instances skeletal rods, differentiated gut and pigment cells appeared. Many of these eggs, however, developed into balls of motile cells which failed to develop further. When in other cases blastulae and gastrulae were formed, however, they were choked with cells and appeared much darker than controls.

For each experiment the number of divisions of the controls and irradiated eggs was plotted against the time since insemination as in

Fig. 1, an example of a dosage series at λ2804A. The dosage in ergs per square millimeter for each curve was determined from the intensity readings and added to the graphs. The difference in time of development to the 8-celled stage between the control and the irradiated eggs was determined from the graph. The retardation of this third cleavage was then plotted against the log of the dosage (in ergs/mm.^{2}) for each of the wave-lengths to obtain a comparison of the efficiency of the various wave-lengths in retarding cleavage. The data are given in Fig. 2. The third cleavage was chosen for the comparison because (1) it is the midpoint of the developmental curves obtained; (2) it can be determined with greater accuracy than later cleavages; (3) it probably occurs after the latent period lag and by the time the effects of the irradiation have had full opportunity for expression. While the data are fairly homogeneous there is variability as detected in the spread of points on each side of the curves. It is possible that eggs from different females react to variable degrees. Part of the variation is undoubtedly

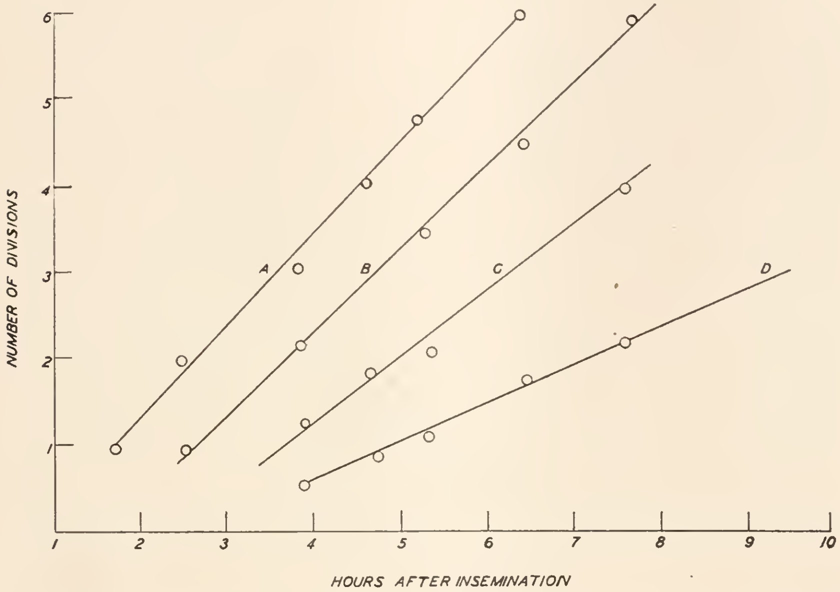

FIG. 1. Retardation of cleavage by irradiation with λ2804A, intensity 2.86 ergs/mm.^{2}/sec.

A. Control.

B. Dosage 183 ergs/mm.^{2}; retardation at 8-cell stage 1 hour and 8 minutes.

C. Dosage 1244 ergs/mm.^{2}; retardation at 8-cell stage 2 hours and 42 minutes.

D. Dosage 3090 ergs/mm.^{2}; retardation 5 hours and 48 minutes.

due to temperature changes and temperature differences for different experiments (T = 15 ± 1° C.). It is possible that minor errors in estimates and in other experimental procedures add further to the spread.

Examination of Fig. 2 discloses that the order of efficiency of the wave-lengths used, on the basis of incident energy, from the most effective to least is (1) 2804A, (2) 2537, 2654, 3025, (3) 3130 and (4) 3660. The difference between (1) and (2) is not large; the difference between (2) and (3), however, is great and between (3) and (4) very great. The difference in efficiency at the different wave-lengths may be due to the differences in absorption at the various wave-lengths. According to the Grotthus Draper law, only absorbed light is effective in promoting a photochemical reaction. If there is to be exact proportionality between absorption and effect, we should expect that the absorption at λ2804A would be about double that at λ2537, 2654 and 3025A and about 20 times that at 3130A.

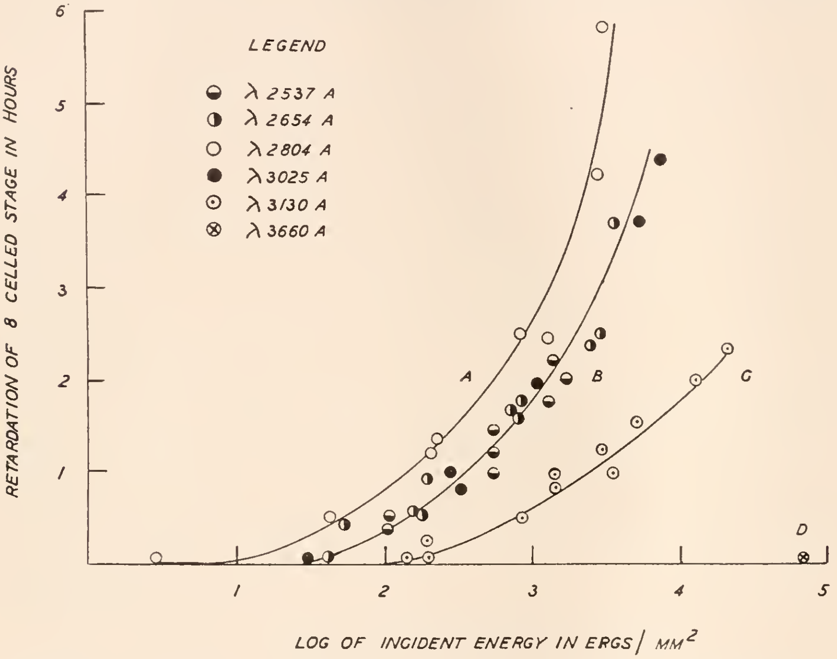

Fig. 2. Difference in efficiency of various wave-lengths in retardation of cleavage.

A. Curve drawn through points for λ2804A.
B. Curve drawn through points for λ's 2537, 2654, and 3025A.
C. Curve drawn through points for λ3130A.
D. λ3660A.

Vlès and Gex (1928, 1934), using a quartz microscope and a spectroscope, measured the extinction of light by single eggs of the sea urchin, *Paracentrotus lividus*. They found for the fresh unfertilized egg a strong absorption band below λ2900A with a maximum at about λ2800A. At λ2537 and λ2654A the absorption was almost as great (the difference being of the order of one per cent), at λ3025A it had fallen off some 20 per cent, and only a little more to the beginning of the visible where another drop occurred. The fertilized egg was observed to have an absorption spectrum similar in general to that of the unfertilized egg (1928). Considerable variation was noted from egg to egg.

Unfortunately comparable equipment was not available to check the extinction by the eggs here used. However, the method previously used for paramecia was employed (Giese and Leighton, 1935*b*), readings of the galvanometer being taken first with the cell empty, then filled with unfertilized eggs, and the eggs counted. Three and in some cases four or five measurements were made at each wave-length, after several preliminary trials in which the eggs were not counted, and the averages are given in Table II. Considerable variation was observed for different samples of eggs. While the experimental error is large, as indicated by the standard deviations, the order of magnitude of the extinction is indicated, and such large differences as between λ2537 and λ2804A are significant. In all cases the differences of the averages of the groups from one another are many times the probable error, which is generally less than ±0.5. In contrast to the data of Vlès and Gex, these data indicate a maximal extinction at λ2537A. Vlès and Gex found in most cases a decrease in absorption by normal eggs at this wave-length although in some of their cases this was not true. For eggs undergoing cytolysis due to dilution of sea water with distilled water they found a rise at λ2537A. That the method here used is not without merit is indicated by measurements on extinction of the ultra-violet light by sea urchin sperm. It was found that the maximum extinction occurred at λ2654A, a considerable decrease occurring at λ2804A, which is well in agreement with what might be expected of a cell made up mainly of nucleoproteins. However, it must be remembered that only part of the extinction is due to true absorption, the rest being due to scattering. With the experimental method here used the scattering would probably be a greater source of error than with the method employed by Vlès and Gex. Scattering varies inversely with some power of the wave-length as well as with the change in refractive index with wave-length (Teorell, 1930). If the change in the refractive index were negligible and the approximate value of the change in scattering with wave-length found for *Paramecium* were to be used here (Giese and Leighton, 1935*b*), one could not explain the high extinction value at λ2537A as due entirely to a higher scattering. On the other hand, it is possible that the refractive index changes suddenly and that a corresponding increase in scattering occurs. The high value at λ2537A may, however, indicate a high absorption. Gates (1930) observed a higher absorption value at λ2537A than at λ2804A for both *S. aureus* and *B. coli*.

It is probable that in both sets of data the scattering masks the true absorption, but it is unlikely that the scattering is so great as to completely disguise differences of the order of magnitude needed to explain completely the differences in action of the different wave-lengths. It therefore seems more probable that it is not total absorption but rather selective absorption by some sensitive material which determines destructiveness of the rays. On this basis the substance absorbing λ2804A is most likely the sensitive substance, for approximately equivalent absorption is found at λ2654 and λ3025A, but their efficiency is less than that of λ2804A. The low efficiency of λ2537A may be due to the fact that radiations of this wave-length are so strongly absorbed at the exterior of the cell that they do not reach the seat of the sensitive material (Sonne, 1929).

TABLE II

Extinction of ultra-violet light by sea urchin eggs

λ in A	$1 - I/I_0 \times \frac{1}{A_e} \times 100$	λ in A	$1 - I/I_0 \times \frac{1}{A_e} \times 100$
2537	105 ± 5.50	3130	64.73 ± 8.00
2654	71.3 ± 4.40	3660	64.53 ± 9.32
2804	76.3 ± 7.78	5844	41.17 ± 4.21
3025	76.06 ± 5.54	5904	25.02 ± 2.44

I_0 = intensity of incident light, I of transmitted light.
A_e = fraction of the cell bottom occupied by eggs.
The whole expression gives the percentage of light incident upon the egg which is extinguished.

Considering the high extinction values, the efficiency of λ3130 and λ3660A is remarkably low. At λ3130A the efficiency is about one-eighth that at λ2537, λ2654 and λ3025A. At λ3660A it is exceedingly small, as even after a dosage of 74,500 ergs/mm.^{2} the eggs showed such slight indications of retardation that accurate measurement of the delay in cleavage could not be made.

DISCUSSION

The failure to obtain acceleration by irradiation with either the lethal region in dosages below the retarding dose or by the non-lethal region of the quartz ultra-violet indicates that either (1) this part of the spectrum is ineffective for acceleration, (2) the dosage range was not the proper one, (3) sea urchin eggs are unfavorable material. It is possible that the sea-urchin eggs are already cleaving at a maximal rate and so the normal cleavage rate cannot be significantly accelerated at a given temperature.

The low retarding efficiency of the long wave-lengths of ultra-violet, 3130 and 3660A, is not surprising on the basis of previous investigations (Gates, 1929; Oster, 1934; Giese and Leighton, 1935*a*) which have indicated a very low efficiency of λ3130A in destroying cells. Wyckoff (1932) has shown that 47 times as much energy is necessary to kill 50 per cent of the bacteria at λ3132A as at λ2654A. The λ3660A is generally considered beyond the region of destructive action. On the other hand, Coblentz and Fulton (1924) consider this region in very high dosages bactericidal, and Hertel (1905) maintained that even at λ4400, 5230 and 5580A retardation of division of sea urchin eggs was obtained when the intensity was sufficiently great.

The high efficiency of λ2804A, whatever the absorption may be, is, however, surprising in view of the many investigations in which λ2654A has been found more effective as a bactericidal (Gates, 1929; Wyckoff, 1932; Ehrismann and Noethling, 1932; and Hollaender and Claus, 1936) and a photolethal agent (Oster, 1934, and Ehrismann and Noethling, 1932, on yeasts; Weinstein, 1930, on paramecia, and Mayer and Schreiber, 1934, on tissue culture). On the other hand, λ2800A has been observed to be more effective than λ2654A in a number of instances (Lassen, 1928; Sonne, 1929; and Giese and Leighton, 1935*b*, on paramecia and Ehrismann and Noethling, 1932, on *B. prodigiosus*).

Since λ2800 is near the middle of the strong absorption band (2700-2850A) of serum and egg albumin (Coulter, Stone and Kabat, 1936) while λ2654 is near the midpoint of strong absorption by thymus nucleic acid and some nucleoprotein derivatives (Heyroth and Loofbourow, 1933) a decision between the two is of importance to the theory of the mode of action of the radiation. Gates (1929) and others following him have postulated that since maximum efficiency in lethal action on bacteria is in the vicinity of λ2654A and since this is the seat of the maximal absorption by nucleoproteins the lethal effect is mediated by absorption of the radiations by the sensitive nucleoproteins. For retardation of the eggs, however, it seems as if the region of maximal efficiency is that absorbed by the proteins of the cell other than nucleoproteins. This suggests that retardation of cleavage may be brought about not so much by an effect upon the nucleus as by interference with some part of the cleavage mechanism. The relative inefficiency of λ2537A which is absorbed strongly by the surface would tend to exclude the surface as the only or the major seat of action. Just which part is affected remains for further experimentation to discover.

SUMMARY

1. The effects of a series of dosages of known intensities of monochromatic quartz ultra-violet light upon fertilized eggs of the sea urchin *Strongylocentrotus purpuratus* were studied.

2. Even at the highest dosages of λ3660A used the eggs cleaved at just about the natural rate of controls; λ3130A produced more pronounced retardation of cleavage; λ2537, λ2654 and λ3025A were about equally effective, and λ2804A most effective of all.

3. No signs of definite and marked acceleration were observed at any of the wave-lengths for any of the dosages employed.

4. The destructive effect may be mediated by some substance most sensitive to λ2804A.

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MONOCHROMATIC ULTRA-VIOLET RADIATION AS AN ACTIVATING AGENT FOR THE EGGS OF ARBACIA PUNCTULATA

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It has been known since 1914 that *Arbacia* eggs can be activated by the radiation from a quartz mercury vapor lamp. Loeb (1914) reported membrane elevation in unfertilized sea-urchin eggs. He obtained cleavage in these same eggs by a subsequent treatment with hypertonic salt solution. The occasional production of parthenogenesis by ultra-violet radiation alone has been reported by Lillie and Baskervill (1922), Tschahotine (1921) and others.

We observed during the summer of 1936 that the radiation from a very intense mercury vapor lamp would activate readily the unfertilized eggs of *Arbacia punctulata*. During the summer of 1937 we have been able to obtain activation in *Arbacia* eggs with monochromatic radiation and found that certain wave-lengths in the ultra-violet region are highly effective in producing parthenogenesis.

Experimental Technique

Experiment with Polychromatic Radiation

An intense beam of radiation from a vertical water-cooled, high pressure quartz capillary mercury vapor lamp (Daniels and Heidt, 1932; Hollaender and Stauffer, 1933) was made to cover by means of a right angle quartz reflecting prism and a quartz condensing lens a horizontal area of 2 by 18 mm. *Arbacia* eggs freshly removed from the ovaries and washed were covered to the height of about 3 mm. with sea water in Syracuse watchglasses, and moved through the beam in such a manner that the dishes were exposed for about 1 to 10 seconds, each egg thus receiving only a fraction of a second of exposure. The eggs in dishes which had received the appropriate quantities of radiation (3 to 5 seconds/dish) showed up to 90 per cent cleavage 3½ to 5 hours after exposure. The activation was not interfered with when the infra-red radiation was removed by means of a water filter. The removal of the ultra-violet radiation below 3000 A by means of an appropriate glass filter inhibited all activation. This definitely located the region in that part of the spectrum between 2000 and 3000 A.

Experiments with Monochromatic Radiation

The next step was to employ monochromatic radiation. We used for this purpose a technique similar to one described previously (Hollaender and Claus, 1936), with certain modifications. A diagram of the experimental arrangement is given in Fig. 1. *A* is the light source (water-cooled high pressure quartz capillary lamp, loc.) using the current from a 500 volt d.c. supply, burning on about 350 volts and 3.5 amperes.[1] *C* are quartz condensing lenses. *D* are quartz prisms mounted on an adjustable turn-table. *E* is a right angle quartz reflection prism mounted in such a manner that it can be turned through an angle of 90°. This makes it possible for the reflected beam to cover a horizontal area of the exposure dish or the vertical receiving surface of the thermopile. *F* is a vacuum thermopile in casing with quartz window connected with a high sensitivity galvanometer (not shown in diagram). The sensitivity of the thermopile was determined against a Bureau of Standards "standard lamp." *G* are two platforms rotated by a small motor (not shown) at identical slow speed. *H* are small

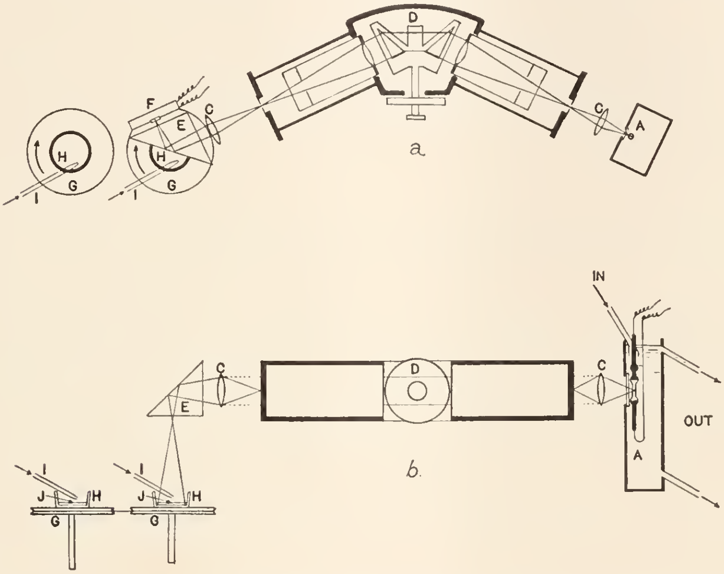

Fig. 1. Diagram of experimental arrangement for the exposure of *Arbacia* eggs with monochromatic radiation. *a*, horizontal, and *b*, vertical cross section. For details see text.

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[1] Special thanks should be given here to Mr. Boss and Dr. Pond of the Marine Biological Laboratory who made available a rectifier-transformer delivering the necessary high voltage.

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(33 mm. diameter) Stender dishes each fastened to the center of a platform. *I* are the tapered outlets which direct a slight stream of air against the surfaces of the liquids in *H*.

For each experiment, the eggs of one *Arbacia* were washed, and about 300 to 600 eggs placed in each Stender dish, and covered to the height of about 3 mm. with sea water. The dish to be exposed was fastened to the platform under the reflection prism, the control dish was fastened to the platform to the left. The two platforms (*G*) with the dishes (*H*) were made to rotate at a uniform speed. Slight air streams of the same pressure for both dishes were directed against the outer quarters of the water surfaces. The rotation of each dish had a tendency to move the eggs against the outer rim, and the air stream tended to bring the eggs to the center of the dish. These two opposed forces made the eggs roll in the dish in such a manner that each egg occupied every possible position in the dish many times during the course of exposure, and every section of the surface of each egg received equal quantities of the radiation.

The efficiency of the stirring was checked in the following manner: Uranium glass particles were placed in the dish with sea water, the dish stirred as described above, and the beam of ultra-violet permitted to fall on the dish. The fluorescing moving glass particles illustrated the efficiency of stirring. If this method of stirring is used carefully, slow rotation, not too strong a beam of air, no damage will be done to the eggs. These precautions were necessary since the beam of monochromatic radiation covered only one-fifteenth of the bottom of the exposure dish. No other mechanical stirring device was found which would fulfill the condition outlined without damage to the eggs.

Before the actual exposure was started, the reflection prism was turned 90° so that the beam from the monochromator would fall on the receiving surface of the thermopile (*F*) as shown in the diagram of the horizontal cross section of the apparatus (*a*). After the energy reading was taken, the prism was turned through 90° and the beam made to cover the described area of the exposure dish as shown in the vertical cross section (*b*), having the dishes rotate during the entire time. Most exposures were for 3 to 10 minutes, but exposures as short as 30 seconds or as long as 30 minutes were made occasionally. Energy readings were taken before and after each exposure. If the exposure was longer than 15 minutes, an additional reading was taken during the middle of the exposure.

The effect of radiation of the following wave-lengths was tested: 2260, 2300, 2380, 2480, 2537, 2650, 2805, 2950, 3050, 3150, 3350, and 3650 A. Since activation was produced with whole eggs only by wave-lengths below 2805 A, and especially with radiation of 2260 to 2380, most of the work described concerns itself with these latter wave-lengths.

After irradiation the eggs were removed to larger dishes and covered with more sea water. Usually the eggs were divided into several lots. One lot kept at room temperature for 4 to 5 hours and then 2 drops of 2 per cent formaldehyde per dish added, and the number of cleaved and uncleaved eggs determined. Another lot kept in the refrigerator at about 10° C. for 15 to 20 hours and then the percentage of cleaved eggs determined. It was necessary to keep the eggs at two different temperatures since the laboratory temperature during the summer of 1937 would rise often to 27°, a temperature critical for the development of the eggs. Apparently the cold seemed to be beneficial.

It is known that *Arbacia* eggs can be activated by many chemicals and by certain physical treatments beside radiation. Special care was taken to recognize such effects if they were produced inadvertently, in these experiments, and to avoid the error of interpreting them as radiation effects. For this reason each group of experiments had the following controls:

1. Eggs of each sea urchin used were fertilized with fresh sperm and only those eggs used which when tested gave a high percentage of cleavage.

2. Each group of four to five exposures had one standard control. That is, these control eggs were handled in an identical manner with the exposed eggs with the exception that they did not receive any ultra-violet radiation. Controls included in the lots were kept at room temperature as well as at 10° C. These controls gave a check in regard to mechanical as well as temperature effects.

3. In the course of this investigation, controls of the following type were used, one to each group of experiments. Dishes with the usual quantities of sea water but without eggs were irradiated with the different wave-lengths and given the different times of exposure found effective in the regular tests. After radiation, unfertilized eggs were added to the irradiated water and permitted to stand with the dishes of exposed eggs. With the wave-lengths and the times of exposures used in this investigation, no effect by irradiated sea water on the eggs could be recognized. If the time of exposure was extended for many hours, effects of the radiation on sea water were found (Rakestraw and Hollaender, 1936). But this lies outside the subject of this paper.

RESULTS

The results of a typical experiment are given in Table I. The manner of calculating the incident energy/egg is illustrated in the following protocol.

Table I

Dish	Time of exposure	Incident energy/egg	Log of incident energy	Eggs cleaved : not cleaved	Percentage cleaved
83	30 seconds	0.335	0.525 − 1	3 : 100	3
79	60 seconds	0.67	0.826 − 1	68 : 220	23.8
77	120 seconds	1.37	0.137	107 : 226	32.1
75	240 seconds	2.67	0.426	283 : 73	79.5
73	360 seconds	4.0	0.602	316 : 62	83.5
82	0 (control)	0	0	0 : 252	0
76	240 seconds	2.67	0.426	268 : 31	89.5
78	120 seconds	1.37	0.137	241 : 81	75
79	0 (control)	0	0	0 : 212	0

Protocol of Part 2 of Experiment 28 August 8, 1937. Eggs removed at 10 A.M. washed twice with sea water, some fertilized, cleaved normally. Kept dish in sea water sink until eggs were needed. Standard exposure dish used. Exposed to 2260 A, 37.5 cm. galvanometer deflection, 61.3 ergs/cm. deflection. The beam covered $2 \times 28 = 56$ mm.^{2}, diameter of dish 33 mm., area $= .7854 \times 33^2 = 855$ mm^2. Part of area of the dish exposed $\frac{56}{855} = 15.3^{-1}$. Formula for calculation of incident energy received per egg with diameter of 74μ.

$$\frac{E \times T \times C \times f}{a} = I$$

E = Incident energy in ergs/second $= 37.5 \times 61.3$
T = Time of exposure = 30 seconds
C = Cross-sectional area of the egg $4300.8\mu^2$
a = Area of beam $= 56 \times 10^6\mu^2$
f = Fraction of time each egg exposed $= \frac{1}{15.3}$
I = Incident energy/egg

$$\frac{37.5 \times 61.3 \times 30 \times 4300.8}{56 \times 10^6 \times 15.3} = 0.348 \text{ ergs/egg}$$

The eggs kept in the first six dishes as given in Table I were held at room temperature until 4 P.M. and then formaldehyde added. Duplicate set of dishes kept in refrigerator for 20 hours. Results of 3 dishes kept in refrigerator are given. Eggs in rest of dishes kept in refrigerator used for cytological examination.

Figure 2 illustrates the results of a typical experiment if one wavelength only was investigated, permitting the determination of many points on the curve. One curve is for eggs kept at room temperature and the other curve for those kept at 10° C. It would appear that the eggs in the dishes kept at room temperatures were given the formaldehyde which stopped their development too early. This was unavoidable, since it was not always possible to determine in advance best time of cleavage, and waiting too long at room temperature brought about occasionally cytolysis of the eggs.

There was a considerable amount of variation in the eggs of different females in their sensitivity to radiation. Thus the incident energy of activation at wave-length 2260 A varied between 2.25 to 4.2 ergs per egg for 80 per cent activation. For this reason the activation energy at different wave-lengths was always determined for the eggs from one individual and in no case has the relative energy necessary to produce activation in its dependence on wave-length been any different from that given in Fig. 3. That is, the lowest energy necessary to produce activation was at 2260 A and activation became negligible around 2500 A, even with relatively high energy values. Figure 3 is not as complete as is desirable because the number of exposures that can be handled in a certain time is limited. Complete curves as given in Fig. 2, if obtained for many wave-lengths, require more time than would be safe to keep eggs from one individual.

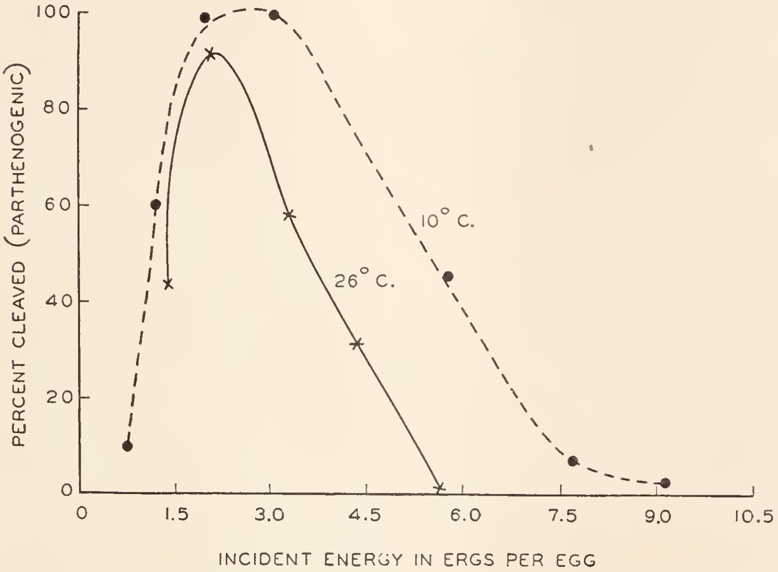

FIG. 2. Graphs showing the percentage of unfertilized eggs cleaved and the incident energy per egg. One set of eggs was kept at 26° C. and one at 10° C. after exposure.

The irradiated eggs showed normal fertilization membranes. But only eggs which showed at least the two-cell stages were called activated. This took usually three to five hours at room temperature. Many of the activated eggs went to further stages of development. Sixteen and 32-cell stages were observed, but some of the eggs cytolyzed after two or three divisions. It was necessary to limit this part of the investigation to the observation of divisions which gave the most reliable counts, i.e., the two-cell stage.

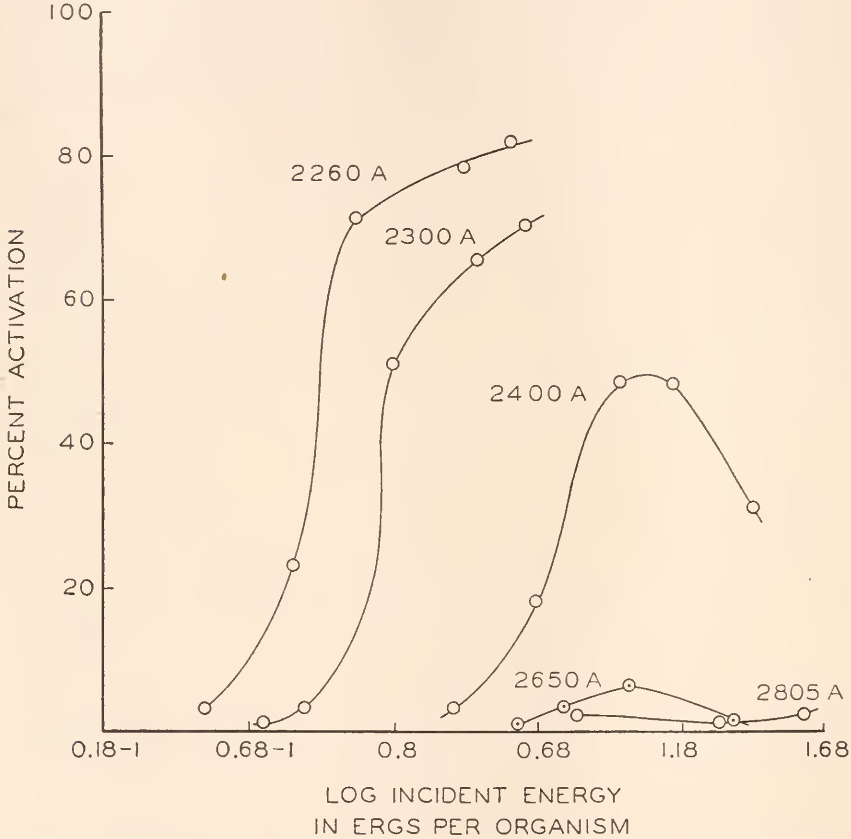

Fig. 3. Graphs showing percentage of the unfertilized eggs activated by monochromatic ultra-violet radiation against the log of the incident energy at five wave-lengths.

Figure 4 illustrates graphically the effectivity of the different wave-lengths in producing activation of *Arbacia* eggs, taking the energy necessary at 2260 A as 100. The absorption spectrum of an egg of *Paracentrotus lividus* as determined by Vlès and Gex (1927) is given. Also, the inactivation spectrum of the virus of typical tobacco mosaic

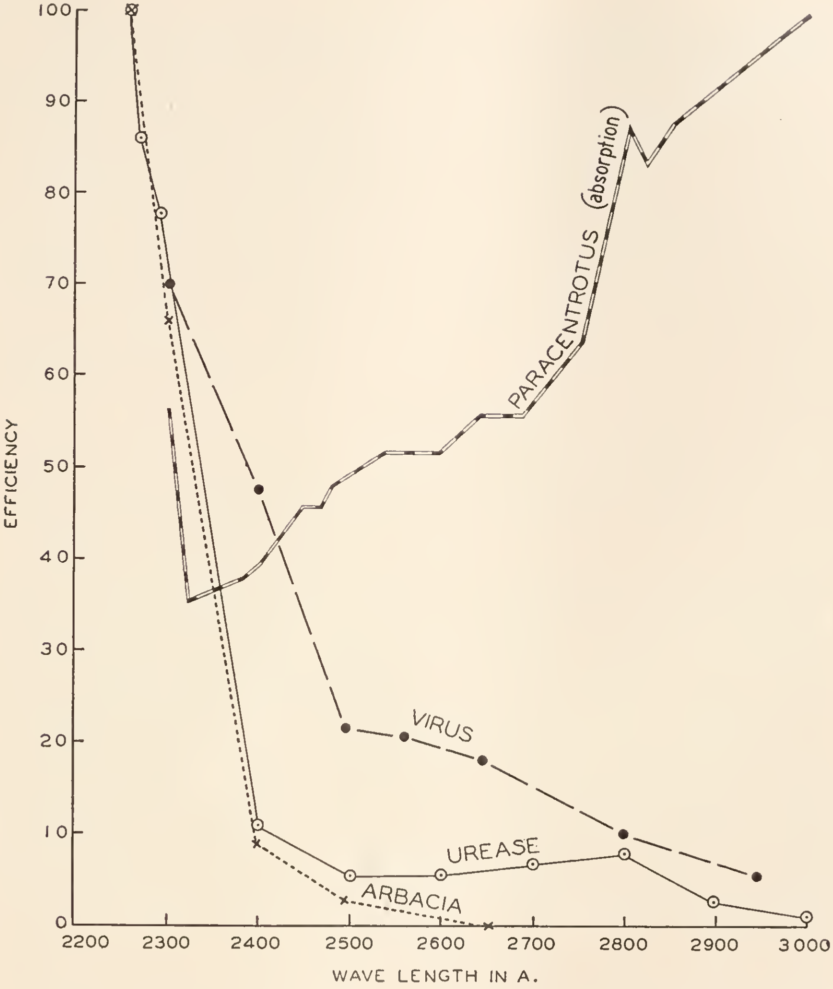

FIG. 4. Summary graph giving (1) the efficiency of the different wave-lengths in producing 50 per cent activation in *Arbacia* eggs; (2) The destruction curve of urease as described by Kubowitz and Haas (1933); (3) the destruction curve of the virus of typical tobacco mosaic (Hollaender and Duggar, 1936); and (4) the absorption curve of one egg of *Paracentrotus lividus* as given by Vlès and Gex (1928).

The data for the relative wave-length dependence of the action of monochromatic ultra-violet radiation on these different agents have been recalculated from the originals to make a comparison possible.

(Hollaender and Duggar, 1936) and the inactivation spectrum of urease (Kubowitz and Haas, 1933—see also Gates, 1934) are given. Data from these different sources have been recalculated to fit into the scheme of this graph and are given only for comparison, i.e., to show the efficiency of different wave-lengths to bring about the investigated effects, and should not be compared for absolute energy values.

The absorption spectrum of an egg of *Paracentrotus lividus* as given by Vlès and Gex is not too helpful as a clue in interpreting the activation (parthenogenesis) spectrum, since it is that of a different species of sea urchin. The taking of such absorption spectrum is difficult and it is felt that it would be very worth while to repeat this work using a somewhat different technique from that used by Vlès and Gex. Coloring matter and other highly absorbing materials contained in the eggs, which vary for different species, mask probably to a large degree the absorption of the essential parts of the egg. However, the rapid increase of the absorption at wave-lengths between 2325 and 2300 (the lowest wave-length tested by Vlès and Gex) indicates a possible correlation with the activating radiation.

The inactivation spectra of urease and a typical virus have been given since these curves follow closely the activation curve of *Arbacia.* The virus curve has been suggested (Hollaender and Duggar, 1936) as the inactivation curve of a protein. The isolation of a crystalline protein with high virus activity from a typical tobacco mosaic virus suspension has been described by Stanley (1937). All these compounds, i.e., proteins, show their highest sensitivity in the ultra-violet below 2400 A. Nucleic acids have their most pronounced absorption around 2650 A (see also Caspersson, 1937) a wave-length not effective in producing activation of *Arbacia* eggs. It would appear from this that the possibility of the eggs being activated by a mechanism which starts with the absorption of the ultra-violet by nucleic acids is excluded. But not too definite conclusions as to the type of compound can be made from the high sensitivity of the *Arbacia* at the short ultra-violet, since besides proteins many other types of organic compounds also increase absorption at these short wave-lengths. We know very little of the effect of monochromatic ultra-violet radiation on proteins, especially the effect of energies which have been used in these tests.

Cytological observations in connection with this work are given in a separate paper (Harvey and Hollaender, 1938).

I wish to thank Dr. E. B. Harvey for her coöperation throughout the course of these experiments and for her criticism of this paper, the University of Wisconsin for placing laboratory space at the Marine Biological Laboratory at our disposal, and the Radiation Committee, National Research Council, for the supply of equipment.

SUMMARY

1. A method for the effective irradiation of *Arbacia* eggs with measured quantities of monochromatic ultra-violet radiation is described.

2. The activation (parthenogenesis) of *Arbacia* eggs by polychromatic and monochromatic radiation is demonstrated.

3. The energy requirements and wave-length dependence of activation have been worked out. The wave-lengths most effective are in the shorter ultra-violet.

4. The wave-length dependence of the activation curve of *Arbacia* is compared with the inactivation curves for urease and the virus of typical tobacco mosaic.

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PARTHENOGENETIC DEVELOPMENT OF THE EGGS AND EGG FRACTIONS OF ARBACIA PUNCTULATA CAUSED BY MONOCHROMATIC ULTRA-VIOLET RADIATION

ETHEL BROWNE HARVEY AND ALEXANDER HOLLAENDER

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Normal unfertilized (uncentrifuged) eggs of *Arbacia punctulata* can be activated by ultra-violet radiation, either by the full arc for 1/10 to 10 seconds or by monochromatic radiation of 2260–2480A for two to eight minutes. The method has been fully described in the preceding paper in this journal (Hollaender). The eggs throw off a normal fertilization membrane and pass through several cleavages (Photographs 1–5). The cleavages are much delayed in comparison with normal fertilized eggs[1] just as they are after treatment with other parthenogenetic agents, such as hypertonic sea water. In some experiments, with the optimum treatment, all the eggs are activated and after five hours, 80 per cent have cleaved (Photograph 1); frequently the first cleavage gives two unequal cells (Photograph 2). In many eggs a second cleavage follows in an hour; it may be asynchronous in the two blastomeres resulting in a 3-cell stage (Photograph 3), or synchronous giving a typical 4-cell stage (Photograph 4). Further cleavages result in 8 to 16-cell stages (Photograph 5), but no further development was observed.

Whole eggs which have become stratified and elongated by a strong centrifugal force (10,000 × *g* for 3 minutes) are activated by the same radiation as the uncentrifuged eggs; they form fertilization membranes following the irregular contour of the egg, just as similar fertilized eggs do, or as do those artificially activated by hypertonic sea water. About 50 per cent of the eggs cleave, in about the same time as the uncentrifuged—that is, after four to five hours. The first cleavage plane usually comes across the short axis of the elongate egg (Photograph 6) just as it does in fertilized or other parthenogenetic eggs of the same shape (E. B. Harvey, 1932, 1936). When the centrifuged egg is spherical or nearly so at the time of radiation, the cleavage plane tends to come in through the equator, just as in normal fertilized eggs. The centrifuged eggs which have been radiated pass

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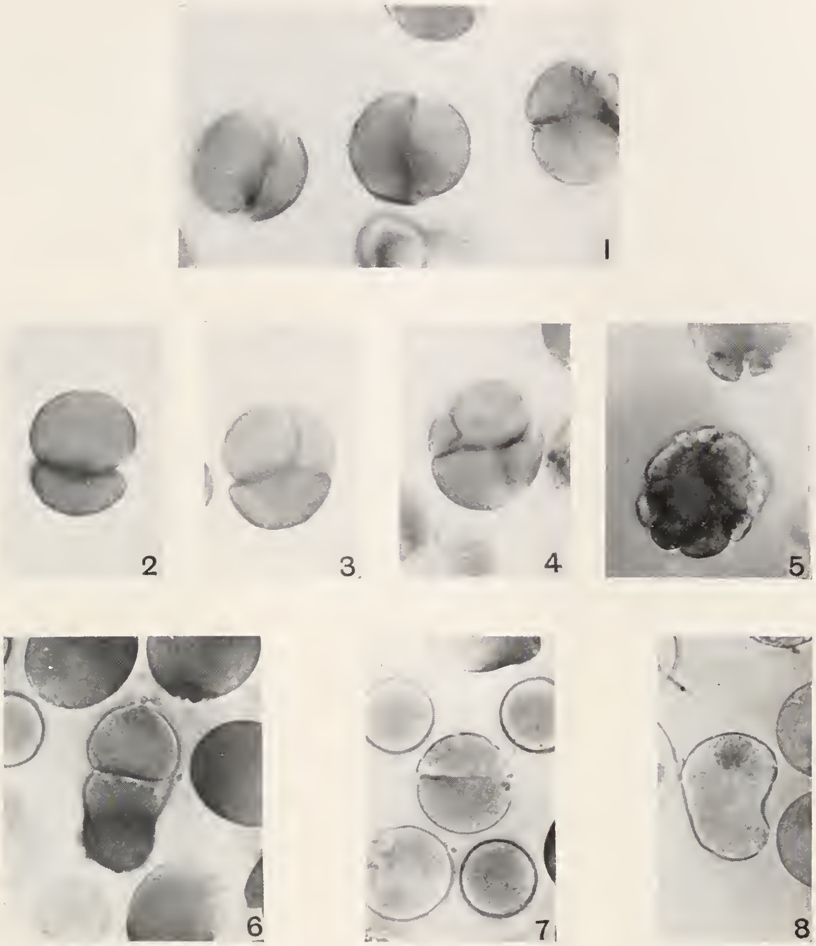
[1] First cleavage of the normal fertilized egg takes place in 50 minutes at 23°.

PLATE I



Photographs of living *nucleate* eggs, taken with a Leica camera and water immersion lens.

1. Unfertilized uncentrifuged eggs, six hours after radiation of 2380A for five minutes. All eggs in field cleaved.

2. Same, 2-cell stage unequal. Cf. Photographs 20 (red half) and 26 (yolk quarter).

3. Same, seven hours after radiation. Three-cell stage. Cf. Photographs 21 (red half) and 27 (yolk quarter).

4. Same, 4-cell stage. (Cf. Photograph 28.)

5. Same, eight hours after radiation. Sixteen-cell stage. Cf. Photograph 23 (red half).

6. Centrifuged whole egg, six hours after radiation with full arc five seconds. Two-cell stage. Cf. Photographs 30, 31 and 36 of E. B. Harvey, 1936.

7. White half-egg, same treatment. Two-cell stage.

8. Another white half-egg, five hours after radiation. Nuclear membrane has just broken and egg is somewhat amoeboid.

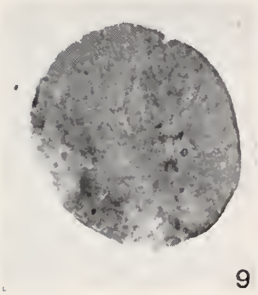
through several cleavages, but stop at about the same stage as the uncentrifuged.

The nuclear changes leading up to first cleavage can be observed in the living egg, especially in the centrifuged egg. After radiation, the usual nuclear changes take place, the enlargement of the nucleus, breaking of the nuclear membrane and the formation of the amphiaster; it is a perfectly normal cleavage in many observed cases. Prepared sections of later stages (8 hours after activation) of radiated uncentrifuged eggs showed some normal prophase, metaphase and anaphase figures (Photographs 9, 10 and 11). Typically there are no asters at the poles of these spindles (Photographs 10, 11) but occasionally they are well-formed (Photograph 9); and large asters may occur independently of chromosomes, in the cytoplasm (Photograph 17). Spindles without asters are characteristic of somewhat later cleavages of the normal fertilized *Arbacia* egg when the cells are very small, but in the early stages corresponding to those pictured here, the asters of the normal dividing egg are very conspicuous. Photograph 12 shows the quite normal-looking nuclei resulting from the first division of a radiated egg. Together with the fairly normal mitotic figures, and in much greater abundance, are abnormal figures. The most characteristic feature of the radiated eggs is the occurrence of multipolar spindles, each spindle with its chromosome plate. The axes of the spindles may be parallel (Photographs 13, 14) or they may lie at any angle with regard to each other, forming a rather confused figure (Photographs 15, 16). The chromosome number appears to be sometimes haploid and sometimes diploid in the more simple type of spindle, but is very large in the compound spindles, and judging from the formation of these spindles should be, at least in some cases, tetraploid (Photograph 14).

White half-eggs, obtained by breaking the whole eggs into two parts by centrifugal force,[2] are also activated by ultra-violet radiation. These are the lighter halves and contain the female nucleus. When activated, they form fertilization membranes, and usually after five hours cleave equally since they are spherical (Photograph 7), just as do white halves which are fertilized. Subsequent cleavages have given 8-cell stages, but no further development. These white half-eggs are much more sensitive to the same amount of ultra-violet radiation than are the whole eggs, normal or centrifuged. With the optimum treatment, only 5 per cent of the eggs had cleaved after five hours; those radiated slightly longer were all cytolyzed after five hours, and those radiated slightly less were not activated. The

[2] For a description of the method, see E. B. Harvey, 1932 and 1936.

PLATE II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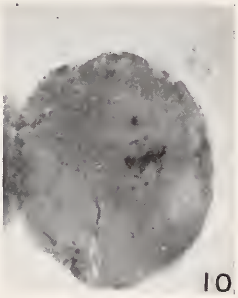
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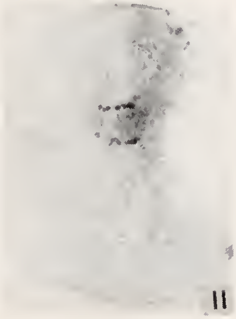
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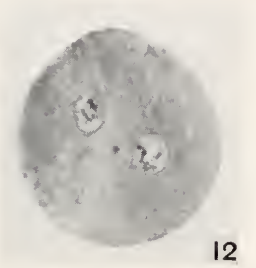
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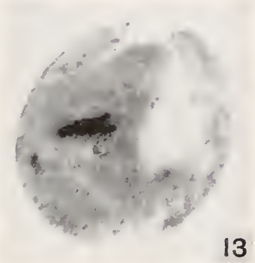
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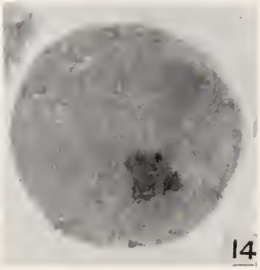
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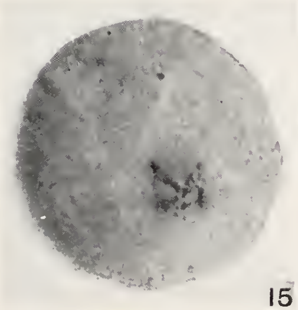
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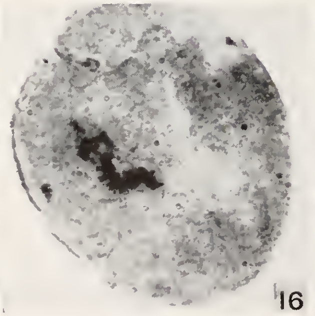
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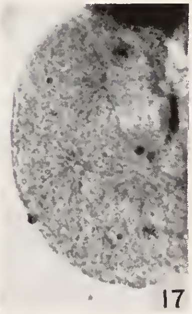
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Photographs of prepared and stained sections of whole uncentrifuged eggs, taken with Leica camera and oil immersion lens. The eggs were fixed seven hours after radiation with full arc for five seconds.

9. Normal prophase with asters.
10. Normal metaphase, but no asters.
11. Normal anaphase, but no asters.
12. Two-cell stage, with normal nuclei.
13. Beginning anaphase, several spindles combined, in same plane; side view.
14. Metaphase plates of four spindles combined in same plane.
15. Metaphase, several spindles combined in various planes.
16. Same, polar view.
17. Large cytasters without spindles or chromosomes.

"dose" is rather critical. There is a tendency for the white half-egg to become aspherical and amoeboid (Photograph 8).

Red half-eggs, the heavier halves obtained by breaking the whole egg into two parts by centrifugal force, do not contain a nucleus, nor any part of it; the nucleus goes invariably into the lighter white halves. The red half-eggs are, nevertheless, activated by ultra-violet radiation. They are activated by the same radiation as the whole eggs and also by a band of longer wave-length, 2650–3300A for four to twelve minutes, a treatment which does not affect the whole eggs and white halves, but does affect the yolk and pigment quarters. They form a fertilization membrane and a thick ectoplasmic layer (Photograph 18). After an hour and a half, a clear area is observed in the less dense portion, a sort of pool, just as in the red halves activated by hypertonic solutions (E. B. Harvey, 1936, p. 111). In a few cases, large monasters in this area are observed four hours after radiation. These have also been found in prepared sections of the red half-eggs (Photograph 31). A notch frequently appears at the equator of the more spherical red halves indicating the beginning of a cleavage plane (Photograph 19). Two-cell stages of these partheno-

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Plate III

Photographs of living *non-nucleate* eggs taken with Leica camera and water immersion lens, except the last photograph, which is from a prepared slide taken with an oil immersion lens.

18. Red half-egg, six hours after radiation of 2380A for five minutes. Note fertilization membrane and ectoplasmic layer.

19. Red half-egg, seven hours after radiation, full arc 10 seconds. Indentation at equator.

20. Red half-egg, eight hours after radiation, full arc five seconds. Two-cell stage. (Cf. Photographs 1 and 2 of similar *nucleate* egg.)

21. Red half-egg, seven hours after radiation of 2380A for six minutes. Three-cell. (Cf. Photograph 3 of similar nucleate egg.)

22. Same lot. Four-cell.

23. Red half-egg nine hours after radiation of 2380A for six minutes. Eight to twelve-cell. (Cf. Photograph 5 of similar nucleate egg.)

24. Red half-egg seven hours after radiation of full arc five seconds. Amoeboid.

25. Same egg one-half hour later.

26. Yolk quarter seven hours after radiation of 3300A for five minutes. Two-cell. (Cf. Photographs 1 and 2 of nucleate egg.)

27. Same egg one hour later. (Cf. Photograph 3.)

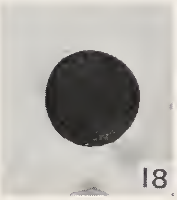
28. Yolk quarter eleven hours after radiation of 2380A for six minutes. Four-cell. (Cf. Photograph 4.)

29. Yolk quarter seven hours after radiation of 3300A for nine minutes. Multi-astral.

30. Pigment quarter (lower cell) seven hours after radiation of full arc five seconds. Amoeboid.

31. Prepared and stained section of red half-eggs seven hours after radiation with full arc for five seconds. Note large aster in center cell and absence of chromosomes and spindles. (Cf. similar nucleate cells of Plate II.)

PLATE III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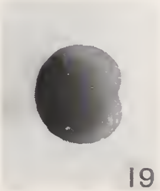
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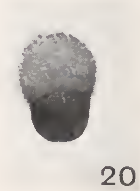
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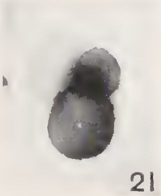
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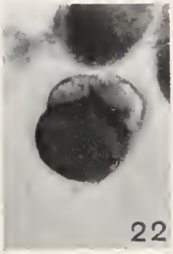
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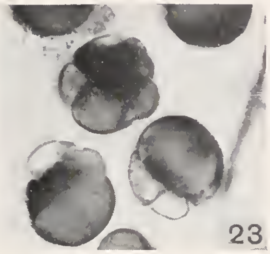
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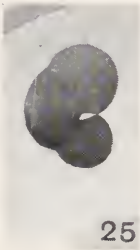
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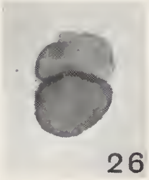
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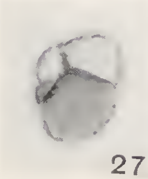
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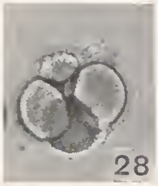
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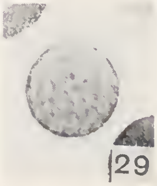
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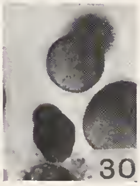
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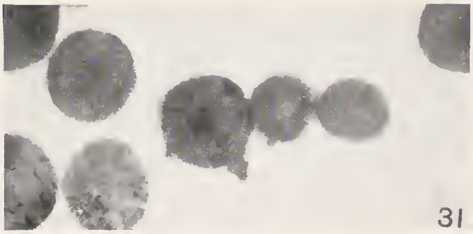
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genetic merogones occur (Photograph 20) and a further cleavage gives a 3- and 4-cell stage (Photographs 21, 22). A 12-cell stage is the latest that has been observed (Photograph 23). Later on, however, some of these eggs are found with many asters, a possible precursor to a blastula and similar to what has been found in red halves activated in other ways. The similarity of the development of the non-nucleate egg with that of the nucleate may be seen by comparing similar stages, such as Photographs 21 with 3, 23 with 5 and 28 with 4. Frequently the red half-egg, after radiation, becomes aspherical and somewhat amoeboid with a tendency to form and split off a lobe from the pigmented end (Photograph 24). This is sometimes completely separated by what appears to be a true cleavage plane (Photograph 25).

The yolk quarter and pigment quarter which are obtained by further centrifuging of the red half, and which are, of course, non-nucleate, are both activated by radiation of the same wave-lengths as the red halves. The yolk quarters cleave several times (Photographs 26–28) to give as many as eight or twelve cells. Multi-astral yolk quarters also occur (Photograph 29).

Pigment quarters, although only one-fourteenth the volume of the whole egg, show evidence of activation in that they form an ectoplasmic layer and become somewhat amoeboid (Photograph 30).

A few clear quarters (nucleate) and granular quarters (non-nucleate) obtained by further centrifuging of the white half-eggs also show signs of activation by radiation. The clear quarters become amoeboid and the nucleus breaks down, and the granular quarter forms a fertilization membrane and ectoplasmic layer. Owing to the difficulty in obtaining these fractions, only a few were radiated, and a thorough investigation was not made.

The foregoing results show definitely that ultra-violet radiation acts upon the whole eggs and their fractions obtained by centrifugal force exactly like other parthenogenetic agents such as hypertonic sea water. The most interesting and important result, however, is that the radiation activates the *non-nucleate* fractions of eggs. The action of the radiation must, therefore, be primarily upon the cytoplasm. The nuclear changes leading up to first cleavage, in the whole eggs, both normal and centrifuged, and in the white half-eggs, seem perfectly normal, but the later spindles and chromosome groups are often quite irregular and abnormal. The conclusion is drawn, then, that the ultra-violet radiation affects primarily the cytoplasm, but there is also an effect upon the nucleus. Whether this is a direct action of the radiation on the nucleus or a secondary effect due to changes first produced in the cytoplasm by the radiation, it is difficult to say. It would rather seem that the effect on the nucleus is secondary since the nuclear phenomena accompanying the *first* cleavage appear to be unaffected.

SUMMARY

1. Ultra-violet radiation either by the full arc for 1/10 to 10 seconds or by monochromatic radiation of 2260–2480A for two to eight minutes causes activation in normal unfertilized eggs of *Arbacia punctulata*, in the centrifuged whole eggs and in the halves and quarters derived from the whole eggs by centrifugal force.
2. Cleavages of the eggs and fractions have been obtained up to a 12- to 16-cell stage.
3. The nuclear phenomena of the nucleate eggs seem to be normal for the first cleavage, but in later cleavages many abnormal mitoses occur, chiefly multipolar and anastral.
4. Fractions of eggs without nuclei are activated by ultra-violet radiation just as they are by hypertonic sea water. These parthenogenetic merogones cleave and go to a 12-cell stage quite similar to that of a nucleate egg.
5. The ultra-violet radiation affects, therefore, primarily the cytoplasm.

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STUDIES IN THE PIGMENTARY SYSTEM OF CRUSTACEA

III. LIGHT-INTENSITY AND THE POSITION OF THE DISTAL RETINAL PIGMENT IN LEANDER ADSPERSUS

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INTRODUCTION

While studying retinal pigment migration in a Mediterranean decapod, *Leander adspersus*, we noticed that the position of the distal pigment cells in the eyes of an animal kept on an illuminated black background was different from that in a shrimp maintained on a similarly-illuminated white background. Although it has been previously shown that the light-adapted condition of the distal retinal pigment in crustaceans is effected by a hormone having its origin in the eye-stalks (Kleinholz, 1936), it has been assumed that the position of the distal retinal pigment at any one time was to be found in either the light-adapted or the dark-adapted condition.

The preliminary observation mentioned above, if verified by further study, would indicate that the position of the retinal pigment could be graded between the limits of dark- and of light-adaptation, and that the gradation could be achieved by varying not only the intensity of light but also the color of the background. This suggests, furthermore, that the pigmentary reactions of the crustacean retina are not entirely determined by the intensity of incident light, but are possibly regulated to some extent by the intensity of reflected light, or by some relation between these two factors. Such a possibility is supported by the fact that Keeble and Gamble (1904), Brown (1936), and Sumner and Doudoroff (1937) have found the chromatic reactions of crustaceans and fishes to be determined by a similar relationship (the intensity of incident light/the intensity of reflected light).

We therefore decided to investigate the relation between light intensity and the position of the distal retinal pigment in the shrimp, *Leander adspersus*.

We are indebted to Professor R. Dohrn for the facilities granted us at the Stazione Zoologica in Naples. One of us (L.H.K.) takes this opportunity to thank the Committee for permission to occupy the Jacques Loeb Memorial Table.

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MATERIALS AND METHODS

To reduce the error introduced by variations in the size of the eye among different individuals, only those shrimps measuring exactly 1.8 cm. from the tip of the rostrum to the posterior margin of the cephalothorax were used in these experiments. Two glass vessels, each about 10 inches in diameter and 5 inches deep, were prepared for the black and the gray backgrounds. The inside of one was covered with a melted mixture of paraffin-wax and lampblack; when this cooled it gave a uniform dull-black background. The gray background was prepared by first applying the black paraffin and then lining the inside of the dish with white-waxed tissue paper.

Ordinary electric-light bulbs of 25, 40, 50 and 100 watts were used as sources of illumination. In most of the series the bulbs were kept at a distance of 22 inches from the bottoms of the containers, but in two cases (series *B*-3 and *A*-4) the position of the electric lamp was adjusted to give ratios which were desired as points on the scale. Exact quantitative measurements of light-intensity were not possible because of the lack of an appropriate photometer. Since only the ratios of incident to reflected light were sought, these were obtained quantitatively in a manner adequate for the purpose of these experiments by using a "Sixtus" photometer (Leica-model). The scale of this meter which expresses light-intensity in terms of seconds necessary for photographic exposure (the lower the numerical value of the reading the higher the intensity of light) gave relative values for the various intensities and backgrounds. The intensity of incident light was measured at the level of the bottom of the vessel. Each value for the intensity of reflected light was the average of 10 readings taken against the bottom of the empty container.

At the start of an experiment, sea water was poured into the dish to a level of 2.5 cm., 5 measured shrimps were introduced, and the temperature of the water taken. Twenty-four hours were allowed for retinal adaptation. At the end of 12 hours the temperature of the water was again taken and the water changed. In no case had the temperature risen more than 1.6°C. at the end of any twelve-hour interval. After 24 hours the eyes were fixed.

The animals were immersed in hot water (80°C.) for 10 seconds to fix the position of the retinal pigments. The eyes were then excised and transferred to a modified Bouin's solution (containing 7 parts of glacial acetic acid) for 24 hours. They were next placed successively in 70 per cent alcohol for 3 hours, in dioxane for 12 hours and in paraffin-wax for 3–5 hours. Before the final embedding the eyes were oriented in the melted paraffin with the dorsal side uppermost (readily recognized by the black pigment-spot located at the junction of the retinal and non-retinal portions of the eye-stalk). Serial sections were cut at 20μ and were mounted unstained. Measurements of the position of the distal retinal pigment were made with an ocular micrometer through the middle of the eye, from the basement membrane to the proximal end of the distal pigment cells in 10 serial sections which included the dorsal pigment spot (Fig. 3). The structure of the eye of *Leander* is similar to that of *Palaemonetes* (Kleinholz, 1936).

In one experiment (Series I) the animals were illuminated from below by a 40-watt lamp, at a distance of 22 inches from the bottom of the clear glass container. The dish used for gray background was inverted over the container.

RELATION OF LIGHT-INTENSITY TO PIGMENT MIGRATION

The data from these experiments are arranged in Table I. Figure 1 shows that, provided the background is kept constant, the amount of migration of the retinal pigment is directly proportional to the intensity of incident light (limited, however, by the total migration possible in passing from complete light- to complete dark-adaptation: about 360μ in the standard-size animals used here). The effect of backgrounds is shown by the different extents to which the distal pigment migrated in animals kept in the black and in the gray containers, but at the same intensities of incident light. The prolongations of the two curves meet

TABLE I

Light-intensity and the position of the distal retinal pigment. Intensity of light was measured in relative rather than in absolute units (see under "Methods"). Series *A* were on the gray background, Series *B* on the black background, Series *D* in total darkness. *I*, intensity of incident light; *R*, intensity of reflected light; the ratio, *I*/*R*, was multiplied to give whole numbers; *P.P.*, distance of the proximal end of the distal pigment cells from the basement membrane in μ, and the average deviation within a series.

Bulb	Series	*I*	*R*	$\frac{I}{R} \times 1000$	*P.P.*
25	*A*-1	0.40	15.0	26	$295\mu \pm 17\mu$
40	*A*-2	0.25	6.3	39	$241\mu \pm 9\mu$
50	*A*-4	0.18	3.5	51	$190\mu \pm 18\mu$
100	*A*-3	0.125	2.0	62	$167\mu \pm 15\mu$
25	*B*-1	0.40	45.0	9	$372\mu \pm 18\mu$
40	*B*-2	0.22	13.0	17	$319\mu \pm 18\mu$
100	*B*-3	0.18	8.1	22	$311\mu \pm 24\mu$
—	*D*	—	—	—	$526\mu \pm 22\mu$

at a point — 515μ — where the positions of the distal pigment should be identical, regardless of the color of the background. Obviously, this theoretical position of the pigment should be found in the retinas of animals adapted to darkness. Measurement of the position of the retinal pigment in 7 dark-adapted eyes gave an average value of 526μ ± 22μ, in good agreement with the theoretical value.

When the data in the last two columns of Table I are plotted (Fig. 2), it becomes evident that, under the conditions of illumination used, the position of the distal pigment is in direct proportion to the ratio, $\frac{I}{R}$.

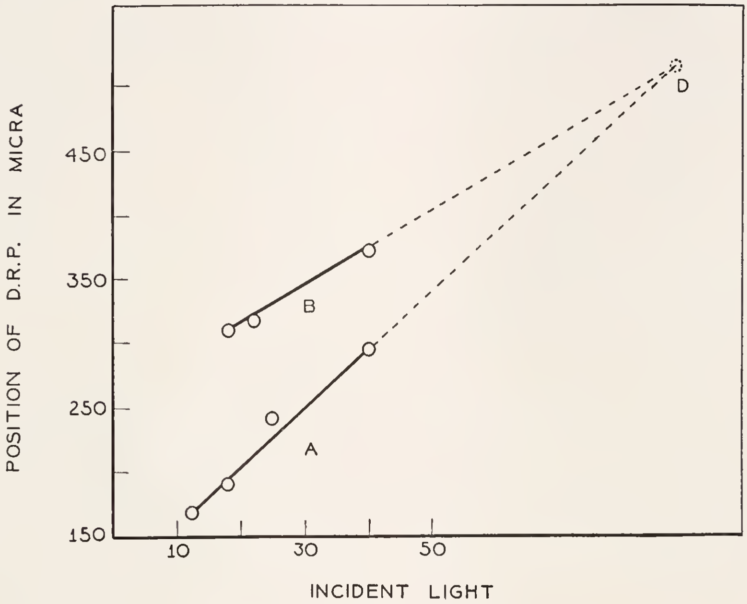

Fig. 1. The relation between intensity of incident light and position of the distal retinal pigment as measured from the basement membrane to the proximal end of the pigment cells. *A*, series on the gray background; *B*, series on the black background; *D*, theoretical position of the pigment in darkness (see text).

Whether the same direct relation would hold for the lower intensities of light, such as used by Brown (1936) in studying the chromatophoral behavior of a teleost, is not known.

Keeble and Gamble (1904, p. 354) found that color reactions were normal in crustaceans which were illuminated from below against white or black backgrounds, and that an implied dorsi-ventrality was therefore "not due to a permanently fixed structural difference in the two sides of the eye" but was produced "by some mobile structure which takes up its position in response to the light conditions."[3] On examining sections of retinas in any particular stage of light-adaptation, it is seen (Figs. 4–7) that the pigment in the dorsal portion of the eye has always undergone a greater amount of migration (toward the basement membrane) than that in the ventral portion. But since migration of the distal pigment is under hormonal control (Kleinholz, 1936), the reason for the unequal position of this pigment is not clear. If the variation in position of the pigment of the dorsal and ventral regions of the retina is constant under all conditions of illumination, the inequality could be attributed to greater sensitivity of the pigment cells in the dorsal portion of the retina to the eye-stalk hormone. If, on the other hand, this difference could be reversed by changing the method of illumination, the above explanation would not be valid; in such an event the greater amount of migration in the portion of the eye most strongly illuminated (toward the source of light) might be due to an additional direct effect of light on those cells. This point was

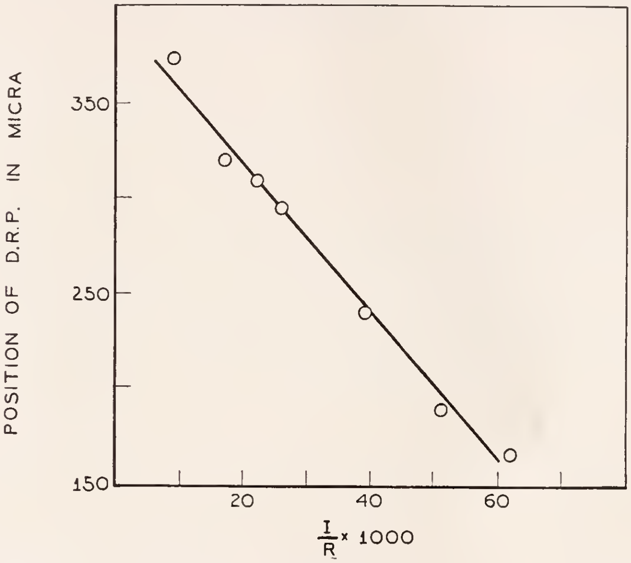

FIG. 2. The relation between position of the distal retinal pigment and the ratio, $\frac{\text{incident light}}{\text{reflected light}}$.

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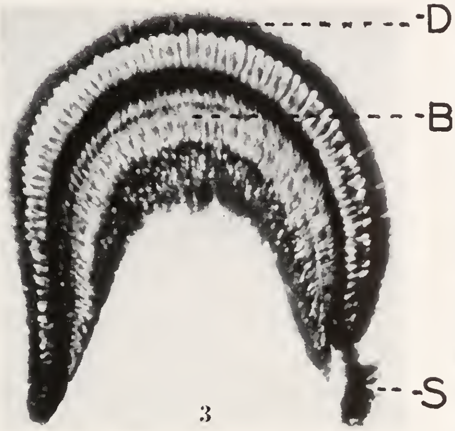
[3] Butcher and Adelmann (1937) reported, however, that illumination of *Fundulus heteroclitus* from below against white and against black backgrounds did not result in the same color changes evoked in illumination from above, therefore implying a fixed structural difference in the retina. This difference apparently lies in the structure and distribution of the rods and cones in the dorsal and ventral portions of the retina (Butcher, 1937).

tested experimentally by subjecting animals to illumination from below against the gray background (see "Methods").

The results from this series are not entirely conclusive. Of 7 eyes studied 3 showed uniform migration of the pigment, the position of the distal cells being the same in the dorsal and ventral halves of the retina; in three others the distal retinal pigment had migrated to a greater extent in the dorsal portion of the eye; and one eye was irregular. Inasmuch as the pigment had migrated to a greater extent in the dorsal

EXPLANATION OF PLATE I



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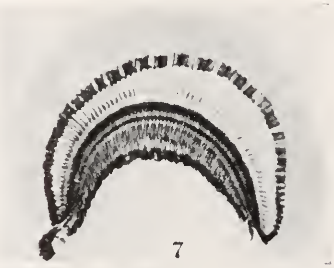
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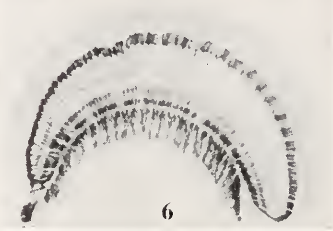
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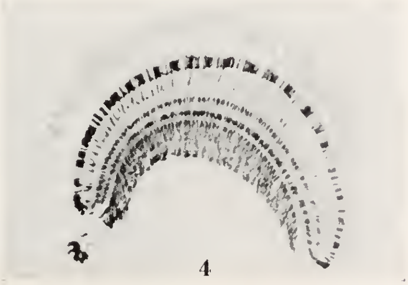
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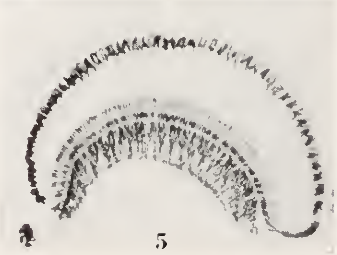
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FIG. 3. An enlarged photograph of a completely light-adapted retina, showing the landmarks used in measuring the position of the distal pigment. *D*, distal pigment cells; *B*, basement membrane; *S*, dorsal pigment spot.

FIGS. 4 AND 5. Retinas from Series *A*-1 and *B*-1 respectively. The intensity of incident light was the same in both cases, but the backgrounds were different. Not only is the position of the distal pigment unequal in the two eyes, but that in the dorsal region of the retina has migrated to a greater extent toward the basement membrane.

FIGS. 6 AND 7. Retinas from Series *B*-2 and *A*-2 respectively.

portion of the retina in the eyes of all animals illuminated from above (except in series *A*-3 where the eyes were completely light-adapted) the fact that in some of the animals illuminated from below migration of the distal retinal pigment was uniform may indicate a slight direct response of those cells to light. This cannot be stated with any certainty, however, and requires further study.

DISCUSSION

It is generally stated that, within certain limits, chromatophoral activity in animals on particular backgrounds is largely independent of the intensity of incident light. Recent studies have been indicating a certain relation between the visual field and the chromatophore response. Thus, Sumner (1933) found that covering the lower halves of the eyes in *Fundulus parvipinnis* resulted in darkening of the animals even on white backgrounds, while darkening the upper half of the field of vision gave only a slight dispersion of melanophore pigment in fishes on white backgrounds. Hanström (1937) obtained similar results in opaquing the dorsal and ventral portions of the retinas of *Palaemonetes vulgaris*. Brown (1936) found that in the minnow, *Ericymba buccata*, "the average diameter of the melanophore masses (therefore the degree of darkening) appears to vary in a directly proportional fashion with the ratio, $\frac{\text{incident light}}{\text{reflected light}}$, which reaches the eyes of the fish."

All these results resolve themselves into a differential stimulation of dorsal and ventral retinal receptors. In *Leander* a similar condition must hold for the migration of the retinal pigment. The migration of the retinal pigment, however, is assumed to regulate the amount of light which arrives at any particular rhabdome. (In dim light the distal position of the pigment allows oblique rays to pass; in bright light oblique rays are excluded, only those rays parallel to the long axis of the ommatidium reaching the rhabdome.) The above experiments, therefore, do not determine whether the different extents to which the retinal pigment migrates in the dorsal and ventral halves of the eye contribute to the differential stimulation of dorsal and ventral photoreceptors or whether they are the result of such a differential stimulation.

SUMMARY

1. On a constant background the amount of migration of the distal retinal pigment in *Leander adspersus* is directly proportional to the intensity of incident light, within the limits of light- and of dark-adaptation.

2. Under the conditions of illumination used in these experiments the position of the distal retinal pigment is in direct proportion to the ratio, $\frac{\text{intensity of incident light}}{\text{intensity of reflected light}}$.

3. In all eyes, except those completely light- and totally dark-adapted, the pigment is found to migrate to a greater extent in the dorsal half of the retina. The reason for this variation in position of the pigment of the dorsal and ventral portions of the retina is not clear.

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INFLUENCE OF ASSOCIATION ON THE SEXUAL PHASES OF GASTROPODS HAVING PROTANDRIC CONSECUTIVE SEXUALITY

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The studies of Orton (1909, 1912, 1922), Kleinsteuber (1913), Giese (1915), Gould (1917*a*, 1917*b*, 1919), Coe (1935, 1936, 1938*a*, 1938*b*), Ishiki (1936) and others have shown the occurrence of protandric consecutive sexuality in all members of the pectinibranch family Calyptraeidae (or Capulidae) which have been investigated hitherto, making it appear probable that this type of sexuality is characteristic of the entire family.

The conditions which inaugurate the change from male to female phase, however, have not yet been fully elucidated. Moreover, Gould (1919) concluded that the development of the functional male phase in *Crepidula plana* depended upon the secretion of a masculinizing substance by an older individual with which the sexually immature or "neuter" individual was associated. Actual contact was deemed unnecessary, since it was assumed that the secretion could pass through the water. This conclusion has been widely quoted in the textbooks, although it has been shown recently (Coe, 1935, 1936) that solitary or isolated young of that species may under favorable conditions assume the functional male phase when living entirely isolated from other individuals. Nevertheless, the masculine characteristics when already established may be accentuated and prolonged in that and other species by the association of the male with a female.

Ishiki (1936) concluded that in both *Crepidula walshi* and *C. aculeata* some stimulation of a chemical nature "is carried toward the small individual from the female" which prolongs the male phase as long as association with the female continues. The present writer (1938*a*), on the other hand, discredits the idea of a masculinizing hormone passing from the female to the associated male and assumes that the accentuation and prolongation of his masculine characteristics result from hormones secreted within his own body as the result of stimuli received by his nervous system from the body of the female through the sense

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[1] Contribution from the Scripps Institution of Oceanography, New Series, No. 29.

organs in his penis and tentacles. Such sensory stimuli doubtless dominate his autonomic responses during copulation as well.

It has been shown (Coe, 1936) that the three common species of *Crepidula* (*C. fornicata*, *C. convexa* and *C. plana*) on the Atlantic coast respond in a somewhat similar manner to the influences of association, although the individuals of the three species differ so greatly in their habits. It has seemed desirable, therefore, to extend these studies to other species in order to determine more fully, if possible, the essential character of the responses observed in nature and experimentally.

There are some ten or eleven valid species of the genus *Crepidula* reported from the Pacific coast. Four of these (*C. onyx*, *C. lingulata*, *C. adunca* and *C. nummaria*) form the basis for the investigation here reported. Each of these species can be obtained in abundance for experimental studies at all seasons of the year. All of them agree in having protandric consecutive sexuality, but each species differs in the associative behavior of the two sexual phases and in their responses to experimental conditions in the aquarium.

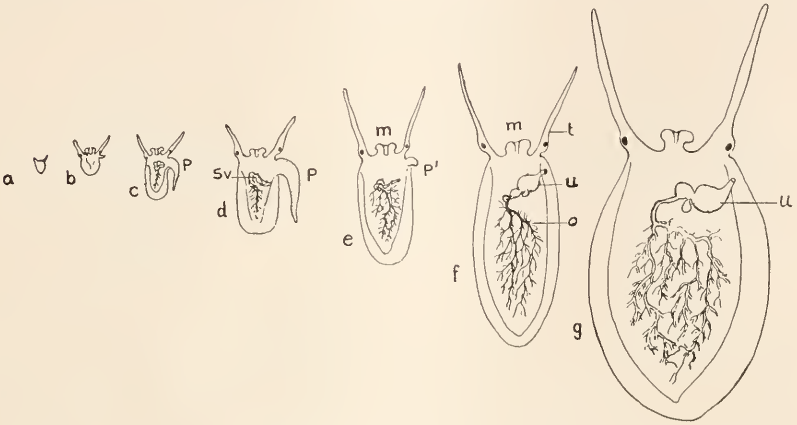

FIG. 1. Successive sexual phases in *Crepidula nummaria*, showing the development and absorption of the penis and the reorganization of the reproductive system preceding the female phase; *a*, recently hatched young; *b*, early male phase, with primordium of penis behind right tentacle; *c*, young functional male; *d*, mated male; *e*, transition stage; *f*, female phase, dwarf form; *g*, female when growing on unrestricted surface; *m*, mouth; *o*, ovary; *p*, penis; *sv*, seminal vesicle; *u*, uterus and seminal receptacle; *t*, tentacle.

In these species, as in others previously studied (Gould, 1917*a*, 1917*b*; Coe, 1935, 1936, 1938*a*; Ishiki, 1936) the male phase becomes functional at an early age, when the body has reached only a small fraction of its definitive size (Fig. 1). After spermatogenesis has commenced a long, muscular penis grows out from the neck behind the right tentacle (Fig. 1). By means of this organ the sperm which have been stored in the seminal vesicles are transferred as opportunities permit to the uterus and seminal receptacle of an older individual in the female phase (Fig. 1).

During the transition from the male to the female phase the residual spermatic cells are cytolyzed and such small ovocytes as may have become differentiated, but not cytolyzed, during the process of spermatogenesis increase rapidly in size and soon begin the deposition of yolk. The gonad thereby changes from a spermary to an ovary. Sometimes this change takes place rapidly but often there is a prolonged period of apparent inactivity, during which the gonadal follicles contain only relatively few ovogonia. In the latter case the body may grow rapidly while the genital duct is being transformed into an oviduct by the absorption of the seminal vesicles and the terminal portion enlarged and extended to form the uterus and seminal receptacle (Fig. 1). Under favorable conditions ovogenesis then follows rapidly.

Accompanying the morphological changes from male to female phase are fundamental alterations of an appropriate nature in the individual's instinctive behavior. The activities characteristic of the male are supplanted by such as are concerned with the formation and attachment of the egg capsules and the provisions for the protection and oxygenation of the developing larvae.

Unfavorable conditions, such as forced movements, accumulation of waste materials, lack of food or of oxygen, abnormal temperature or parasitism, when not fatal, terminate the male phase without the compensatory development of the female characteristics, leading to an essentially neutral sexual condition. If a favorable environment is restored the transformation to the female phase is resumed, but not otherwise. Occasionally the male phase may be aborted and the female functional. Excessive infestation by trematode larvae may also result in the almost complete destruction of the gonad after the female phase has become established.

Since only fragmentary accounts of the biology of these species have been published it seems advisable to discuss each of the four species separately, particularly in regard to the sexual phases and reproductive behavior.

Crepidula onyx Sowerby

(*C. rugosa* Nuttall)

This species closely resembles *C. fornicata* of the Atlantic coast—but now introduced and established on the Pacific coast—both in appearance and in habits. Pyramidal groups of three to sixteen or more individuals are often found in muddy bays. In such groups the basal individual is attached to an empty shell of the same or of some other species of mollusk. This and one or more of the superimposed individuals are usually in the female phase, while transition forms, sedentary males, supplementary males and sexually immature individuals may be superimposed in the sequence named (Fig. 2, *a*).

The supplementary males move about from one female to another, while the sedentary males retain the same position permanently with respect to the immediately underlying individuals. Nevertheless, these sedentary males are capable of sufficient movement to enable them to fertilize females not immediately adjacent. They return to their original positions after copulation has been accomplished or at the approach of daylight, for the crepidulas are largely nocturnal in their activities.

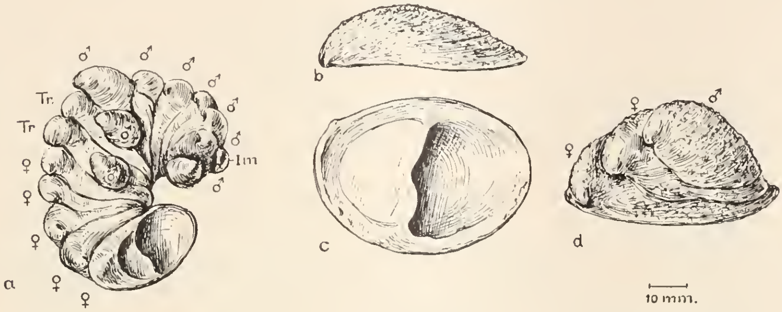

FIG. 2. *Crepidula onyx; a*, typical form, large group consisting of seventeen individuals from intertidal mud flat; the group includes five basal females, two transition individuals and seven superimposed functional males, with two small supplementary males and one sexually immature young (*Im*); *b*, large, isolated female of the roughened, flattened form (*rugosa*); *c*, same from ventral surface of empty shell; *d*, rugosa form, small group consisting of two basal females and one superimposed male. Scale indicates natural size.

Before beginning transition to the female phase the sedentary males often reach a length almost equal to that of some of the underlying females. In such males the transition period is very brief (Fig. 6).

The supplementary and solitary males, on the other hand, as is also the case with many mated males that are isolated experimentally when of small size, have a much longer transition period. During this period they grow considerably but not sufficiently rapidly to equal the sedentary males in size (Fig. 6). Growth also continues after the female phase is reached.

Another habitat form has a flat, oval shell and has been given the varietal name *rugosa* because of the roughened surface (Fig. 2, *b*, *c*, *d*). Individuals of this form occur singly or in groups of two, three or four, usually attached to *Pecten* or other large shells, or to rocks or spiles. These often become much larger than those living on muddy bottoms.

Females may exceed 60 mm. in length, while in mated or associated individuals the male phase may continue until the shell has a length of 35 mm. or more. A large proportion of the males, however, are solitary and transform to the female phase when relatively small. In other habitats only dwarf individuals are found.

In this species the accentuation and prolongation of the male phase in associated or mated individuals as compared with those which remain supplementary or solitary either by chance or by choice is very great (Fig. 6). Individuals of all ages are remarkably hardy, living for many months in the aquarium if sufficient food is available. Isolation of mated males usually, but not in all cases, terminates the male phase and inaugurates the female phase promptly.

Crepidula lingulata Gould

(*C. dorsata* Broderick)

This species likewise occurs in several different forms as the result of various environmental conditions.

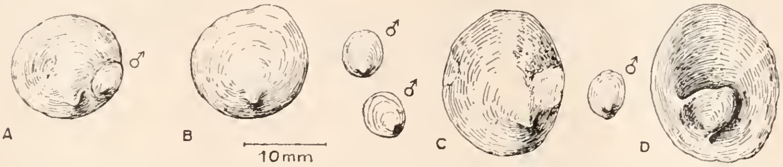

Fig. 3. *Crepidula lingulata.* *A*, mated pair. *B*, female with two not closely associated males. *C*, female with mated male removed, showing "scar" indicating that the male may sometimes remain attached for a considerable period. *D*, shell of female from ventral side to show character of internal partition.

The male phase commonly becomes functional when the young individual has reached a length of 3 to 5 mm., while the transition stages occur at about 8 to 11 mm. The average individual assumes the female phase when about 9 to 12 mm. long, after which growth usually continues until a length of 18 to 20 mm. is reached. An occasional female may be more than 25 mm. long.

It would appear that only temporary matings are the rule in this species since not more than 5 per cent of the mature females observed were accompanied by mated males (Fig. 3) at the time of collection. Only about 5 per cent of the other females showed scars to indicate where the males had been formerly attached (Fig. 3, *c*). All of them, however, had sperm in their seminal receptacles, indicating that temporary matings, presumably nocturnal, had taken place. The males were scattered about in the neighborhood but only occasionally in the immediate vicinity of the females. Consequently in this species no evidence is available as to the effects of association on the sexuality of the male. None of the functional males observed exceeded 12 mm. in length.

CREPIDULA NUMMARIA GOULD

(*C. nivea* C. B. Adams)

This species also is very variable in form as a response to the habitat of the individual. When growing within the narrow space inside an empty shell of *Tegula* or other small univalve, the adults in the female phase are very slender and only 14 to 16 mm. in length (Fig. 4, *a*, *b*). When attached to the surface of a large shell, stone or other solid object, however, the shell becomes broadly oval (Fig. 4, *c*, *d*) or even nearly circular, as was the case with Gould's type specimen, and may sometimes exceed 25 mm. in length.

There can be no further doubt as to the specific identity of the two forms, since the newly hatched young of the slender form have grown into the broad form *nivea* when reared in the aquarium at the Scripps Institution of Oceanography.

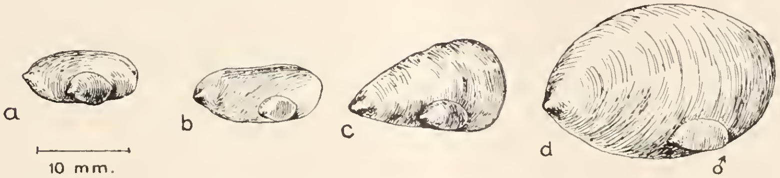

FIG. 4. *Crepidula nummaria*. *a*, *b*, mature females, dwarf form, with mated males, living in small univalve shells; *c*, *d*, same when living on surface which allows unlimited space for growth. Scale shows natural size.

The young often reach the functional male phase within a few weeks after hatching, when the body is less than 3 mm. in length (Figs. 4, 6). Others remain sexually immature until they are 6 to 8 mm. long. The sexual transformation commonly takes place at a length of 7 to 10 mm.; most individuals more than 10 mm. long are in the female phase (Fig. 6). The successive stages in the development and transformation of the reproductive system in the two sexual phases are shown in Fig. 1. The early gonad is less distinctly ambisexual than in the other species investigated, since very few ovocytes become differentiated until toward the end of the transition period.

The males are not usually found in the mating position on the females (Fig. 3) but remain associated nearby or in contact until afforded opportunities for copulation. The influence of association in the accentuation and prolongation of the masculine characteristics becomes evident when series of associated and solitary males are compared (Fig. 6). Unfavorable conditions postpone sexual maturity as well as terminate the male phase and delay the differentiation of the female phase.

Crepidula adunca Sowerby

This species resembles *C. convexa* of the Atlantic coast (but now established on the Pacific coast) in its habits (Coe, 1936).

Conklin (1897, 1898) studied the reproduction and development of this species and Moritz (1938) has described the anatomy of the young male. The mated males retain the mating position for a long period (Fig. 5) but usually leave the female at the beginning of the transition phase.

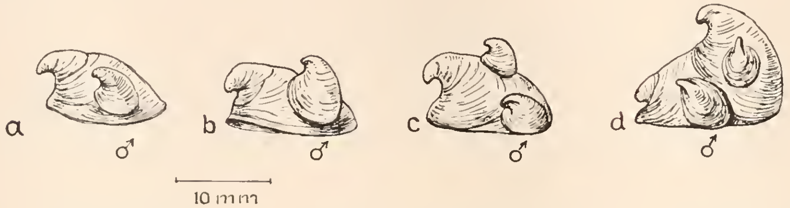

Fig. 5. *Crepidula adunca.* Outlines showing mated females, with associated males; (*a*) female with male of average size; (*b*) female with relatively large male; (*c*) female with mated male and supplementary male; (*d*) two superimposed females each with mated male.

The young may reach the functional male phase within a few weeks after hatching, sometimes when less than 4 mm. in length. The sexual transformation commonly begins when a length of 9 to 10 mm. is reached (Fig. 6). The influence of association or mating on the accentuation and prolongation of the male phase is less evident in this species than in most others that have been studied, although the tabulation of many observations shows a significant difference between solitary and mated individuals (Fig. 6).

Comparison with Other Species

Three of these species, *C. onyx*, *C. nummaria* and *C. adunca*, demonstrate the influence of association on the accentuation and prolongation of the male phase (Fig. 6). In the fourth species, *C. lingulata*, no such evidence has been obtained. In none of these species, however, nor in any member of the family Calyptraeidae thus far investigated, is such association a prerequisite for the functional development of the male phase, but only for its accentuation and prolongation.

The three species of *Crepidula* on the Atlantic coast, *C. convexa*, *C. plana* and *C. fornicata*, show the same influence of association as do the three Pacific coast species mentioned (Coe, 1936, 1938*a*). This is likewise true of the two Japanese species studied by Ishiki (1936) as well as of *Crucibulum spinosum* (Coe, 1938*b*).

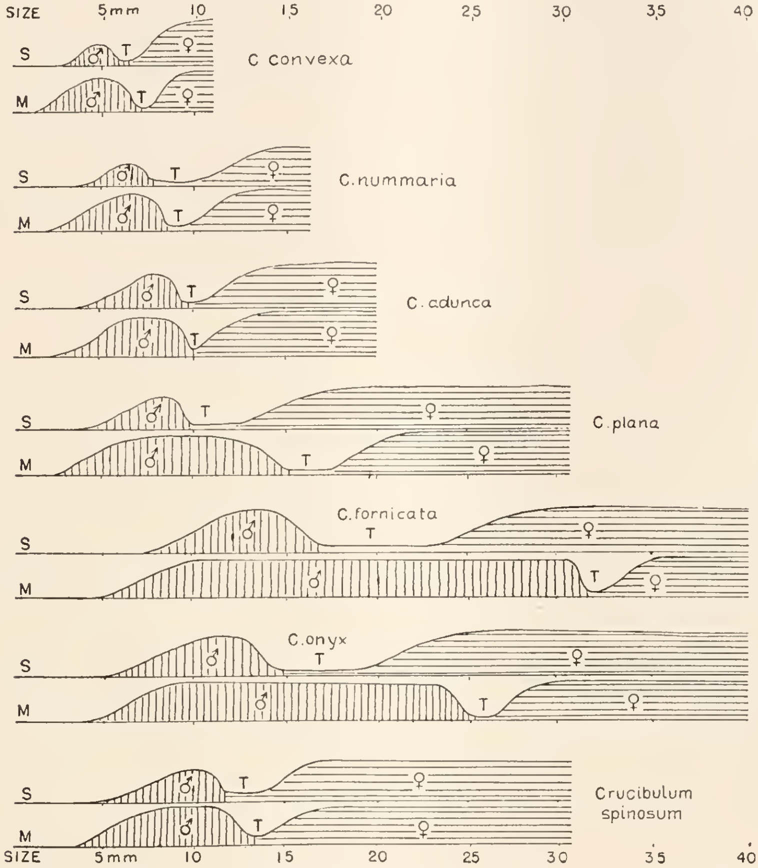

FIG. 6. Diagrams indicating the relative duration of each of the sexual phases in isolated or solitary males (*S*) as compared with associated or mated males (*M*) in six species of *Crepidula* and in *Crucibulum spinosum*. In each case the height of the curve indicates the relative development of the reproductive system. The sizes given indicate the lengths of the shells. For each species it will be noted that the solitary individuals have a briefer functional male phase and a longer transition period (*T*) than the corresponding associated individuals. All the diagrams are drawn at the same horizontal scale, although the heights of the curves are not strictly relative for the different species.

The comparative development of the gonads in the sexually functional and transition phases in solitary and mated or associated males in six species of *Crepidula* and in *Crucibulum spinosum* is shown diagrammatically in Fig. 6. All are drawn at approximately the same scale as regards body length. The accentuation and prolongation of the functional male phase in associated or mated (*M*) as compared with isolated or solitary (*S*) individuals are characteristic for each species. The transition period is longer in solitary individuals.

It will be noted also that the male phase is inaugurated at a smaller size and a presumably younger age in mated than in solitary individuals. It has been assumed that the mated young develop functional maleness earlier as a result of their association but it seems more plausible that those young which reach the male phase earliest are the ones that are most likely to be mated earliest by virtue of the early development of their masculine instincts which lead them to seek the female. Those with less masculine characteristics are presumably more likely to remain solitary (Coe, 1938*a*). Indeed, it seems not improbable that there may also be minor genetic differences in the two groups.

Whether the mating is to be (a) permanent, as is the case with some individuals in *C. onyx* and *C. fornicata* or (b) long-continued, as may occur in *C. convexa*, *C. adunca* and *Crucibulum spinosum*, or (c) highly variable in length, as in *C. plana* and *C. nummaria*, or (d) comparatively brief, as in *C. lingulata*, is also presumably dependent upon genetic differences. Both specific differences and individual peculiarities in instinctive behavior are doubtless involved, as well as environmental influences. Also the question of chance as distinguished from choice must be taken into consideration before a conclusion is reached as to the actual significance of the behavior observed.

With these precautions in mind the nature of the influence whereby the presence of the female accentuates and prolongs the functional male phase may be considered. Experimental evidence indicates that the empty shell of the female does not have the same effect (Coe, 1938*a*). That a masculinizing hormone or other chemical substance passes through the water from the older to the younger individual or from the female to the associated male, as Gould (1917*b*, 1919) and Ishiki (1936) have supposed, seems improbable for the reasons stated in the introduction to this article.

There can be no doubt, however, that the associated male receives stimuli from the female through his sense organs in the penis and tentacles. These sensory stimuli not only affect the behavioristic instincts of the male but they also elicit responses at the time of copulation, resulting in the transfer of sperm to the seminal receptacle of the female. They doubtless also influence such other parts of the male's autonomic system as are concerned with the accentuation and prolongation of his masculine characteristics, presumably through the secretion of hormones within his body.

In the immature male the activation of the spermary and the inauguration of spermatogenesis precede the growth of the penis. Moreover, cessation of active spermatogenesis precedes the absorption of the penis at the termination of the male phase whether such termination be due to isolation, to unfavorable environment or to the internal changes which take place when a certain degree of maturity is reached. Sexual metamorphosis in these mollusks resembles other types of metamorphosis in animals in that the juvenile (male) phase can be abbreviated or prolonged experimentally but the sequence of the sexual phases cannot be reversed. It may be assumed that all these changes are mediated by internal secretions.

It is significant that in these species of *Crepidula* and *Crucibulum*, as well as in other described cases of protandric consecutive sexuality in planarians, nemerteans, oligochaetes, chaetopods, echinoderms' crustacea, pulmonate gastropods, oysters, clams, teredos and other forms the activation of the male constituents of the primary ambisexual gonad always precedes the development of such secondary morphological and behavioristic sexual characteristics as are peculiar to the functional male phase. The external environment in many cases determines the extent of this phase. The termination of the male phase and the metamorphosis to the functional female phase are similarly correlated with the activation of the ovarian constituents of the gonad. Such also occurs in the cases of alternative sexuality, as in the oviparous oysters, and of rhythmical sexuality, as in the larviparous oysters. That these developmental and transformational activities are mediated by the secretion of growth-stimulating substances to which certain localized groups of cells are attuned seems highly probable.

SUMMARY

1. The functional sexual phases of four species of Pacific coast gastropods with protandric consecutive sexuality have been investigated with regard to the conditions which control the change of sexual phase and particularly with respect to the influence of association or mating on the individual's sexuality.

2. In none of these species nor in six species previously studied is association a prerequisite for the development of the functional male phase, although isolation of these males after a prolonged mating period usually terminates the male phase promptly.

3. In nine of the ten species studied intimate association or permanent mating of the young male with one or more individuals in the female phase tends to accentuate the masculine characteristics and to postpone sexual transformation by prolonging the male phase. In five of these ten species the influence of association is very conspicuous; in four it is apparent but not conspicuous; in only one is the evidence inconclusive.

4. There seems to be no evidence that the accentuation and prolongation of the male phase by association is due to any masculinizing chemical secretion or hormone which passes through the water from the body of the female to that of the male, as some investigators have assumed; it seems more probable that stimuli received by the male through the sense organs in his penis and tentacles during association with the female are of such a nature as to influence his masculine characteristics, presumably by the mediation of hormones secreted within his own body.

5. Sexual maturity and sexual metamorphosis in these mollusks, like other types of metamorphosis in animals, are subject to environmental influences and can be accelerated, postponed or inhibited experimentally, but the sequence of sexual phases cannot be reversed.

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PHYSIOLOGY OF REPRODUCTION OF OSTREA VIRGINICA

II. Stimulation of Spawning in the Female Oyster

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Introduction

The second part of the investigations on the physiology of reproduction of the oyster (see Galtsoff, 1938) deals with the conditions which provoke ovulation and the discharge of eggs by ripe females. The work was carried out at the U. S. Fisheries Laboratory at Woods Hole. Live material for the experiments was obtained from Onset and Wellfleet, Massachusetts; Long Island Sound and the Chesapeake Bay. No difference was observed in the reactions of oysters from these three localities.

Ecological literature provides numerous data suggesting a correlation between the breeding periods of various marine animals and sea temperature (Plymouth Marine Invertebrate Fauna, 1904; Lo Bianco, 1909; Zernov, 1913). An attempt to analyze a mass of these observations from a physiological point of view was made by Orton (1920), who grouped all marine organisms into three classes: (*a*) those which breed at a definite temperature characteristic for the species throughout its range of distribution; (*b*) those in which breeding is influenced by a change in temperature, either at the maximum or the minimum for the locality; and (*c*) those which breed the year round. Yonge (1930) has demonstrated that some of the animals of the latter group breed for the greater part of the year not continuously, but periodically. Numerous observations on *O. virginica* (Ryder, 1887; J. Nelson, 1904; Moore, 1907; Churchill, 1920; Gutsell, 1924; Prytherch, 1928; Galtsoff, Prytherch and McMillin, 1930) have shown that this species spawns at rising temperature and therefore should be included in the second group of Orton's classification. Temperature about 20° C. was considered by these observers to be the lowest at which spawning takes place. Nelson (1928*b*) states that "once this 'critical temperature,' 20° C., is reached a 'trigger' mechanism is released which requires some hours for its consummation, and that the speed of the underlying processes is governed by temperature." Ecological evidence upon which this conclusion is based consists in daily records of the occurrence of oyster larvae in plankton, temperature data and weekly examination of oyster gonads. Its inadequacy in dealing with the physiological problem is apparent for neither the act of spawning nor the exact temperature of water, which is supposed to induce it, were observed in the field. Because the daily fluctuations in temperature in shallow water may exceed several degrees, the concept of "critical" temperature in nature has very little meaning unless it is specified whether the daily average or maximum or minimum is considered. Further observations by Nelson (1931) made in England show that *O. virginica* can be induced to spawn at 19.1° C. This throws further doubt on the validity of the view that 20° C. is the "critical" temperature in spawning of this species.

That temperature is not the only factor which governs the propagation of the oyster is shown by the observations of Hopkins (1931) and Loosanoff (1932) in Texas and Virginia. The breeding season in these waters extends over a period of several months during which the temperature remains well above 20° C. Since outbursts of spawning occurring during this time do not coincide with the rise or fall of temperature, other stimuli must be responsible for provoking sexual activity. Inasmuch as field observations fail to throw light on their nature, an experimental investigation conducted under carefully controlled laboratory conditions appears to be more promising.

STIMULATION BY TEMPERATURE

The question whether temperature alone can stimulate ovulation and the discharge of eggs from the pallial cavity of an oyster was studied in the two sets of laboratory experiments designed to determine the effects (1) of a prolonged exposure at increased constant temperature, and (2) of a quick rise of temperature from about 20° C. to 34°–35° C.

In the first series of experiments ripe females, kept in a live car in the bay, were brought to the laboratory and placed in water warmed by means of a no-lag immersion heater, to a desired degree. By using a mercury thermoregulator the temperature was kept constant within ± 0.2°. To avoid the effect of chemical stimulation by sperm which might have been present in the laboratory water supply, all the water was filtered through a 2½ inch layer of asbestos. If the experiments lasted more than 12 hours, running sea water was provided, otherwise the water was aerated and stirred but not changed. The beginning and end of the spawning reaction were determined from the kymograph records of shell movements. The presence of eggs which were collected from the bottom of the tank and kept under observation for

24 hours served as a supplementary proof that spawning took place. In all the cases they were unfertilized and failed to develop, indicating the absence of sperm in the water. The time which elapsed from the moment the oyster was exposed to a given temperature until the beginning of spawning is designated as the latent period of the reaction. The temperature of water in which the oysters were kept before the experiments varied between 19.5° and 20.6° and the range of temperatures to which they were exposed during the experiments extended from 22.6° to 30.0° C. For the sake of convenience the results presented in Table I are arranged in four classes beginning with a temperature increase not exceeding 2.5° and ending with a ten degree rise. Many experiments in which oysters failed to respond to temperature stimulation are not included in this table, which contains only positive results. An examination of column 6 discloses the lack of correlation between the temperature and the latent periods of spawning reactions. Each class of experiments comprise two distinct groups of slow and

TABLE I

Stimulation of spawning by temperature

Experiment number	Date	Temperature ° C.			Latent period	F
		Before	During	Increase		
					minutes	*hrs.×° C.*
437	July 9, 1934	20.6	22.5	1.9	591	19.7
437a	July 9, 1934	20.6	22.5	1.9	659	20.87
399	July 15, 1932	20.0	22.5	2.5	3060	127.50
498	July 5, 1935	20.3	25.0	4.7	35	2.74
498a	July 5, 1935	20.3	25.0	4.7	12	0.94
317	July 7, 1931	19.9	25.3	5.4	250	22.50
433	July 7, 1934	21.4	25.4	4.0	501	33.40
433a	July 7, 1934	21.4	25.4	4.0	500	33.30
321	July 9, 1931	19.9	25.0	5.1	22	1.87
325	July 10, 1931	19.5	24.5	5.0	65	5.42
492	Sept. 5, 1934	20.4	26.0	5.6	71	6.63
432	July 6, 1934	21.0	27.5	6.5	338	36.62
432a	July 6, 1934	21.0	27.5	6.5	340	38.83
318	July 8, 1931	19.9	28.0	8.1	32	4.32
319	July 8, 1931	19.9	28.5	8.6	55	7.88
322	July 9, 1931	19.9	26.0	6.1	257	26.13
320	July 8, 1931	19.9	30.0	10.1	42	7.07
438	July 10, 1934	20.6	30.0	9.4	405	63.45
438a	July 10, 1934	20.6	30.0	9.4	450	70.50
340	July 17, 1931	20.4	30.0	9.6	20	3.20

quick reacting oysters, the latent periods of which at 25° and above, fall either within 12 to 71 or between 250 and 501 minutes. Three experiments carried at 22.5° C. also show two groups of oysters with latent periods of about 600 and 3,000 minutes respectively. By examining the data presented in column 6 of the table, one can notice that the values of latent periods in different groups almost overlap. For instance, the minimum latent period of the first group (22.5°) is 591 minutes as compared with 501 minutes, the maximum latent period of the next higher temperature group. Plotting the latent periods against the temperature results in an erratic distribution of data showing an absence of any definite trend. Similar results are obtained if the amount of heat, *F*, received by the oyster and expressed as a product of the latent period multiplied by the degrees of increase in temperature, is considered. Examination of the last column of Table I shows wide fluctuation in this value and the absence of any definite correlation between it and the latent period.

Since under the conditions of the experiments, negative results have no meaning unless it is proved that experimental oysters were capable of spawning, every female that failed to respond to the increase of temperature was at the end of the experiment stimulated by sperm. Positive reaction was a proof that the female was physiologically ripe and that the lack of the response was due to the ineffectiveness of the stimulus. It was found that thermic stimulation fails in a larger number of ripe females. For instance, of the two females used in experiment No. 399, only one (shown in Table I) spawned at 22.5° C. Thirty-two hours of exposure at this temperature failed to stimulate spawning in the other one, which, however, copiously discharged eggs within a few minutes after the addition of sperm. In another experiment (not listed in Table I) three ripe females were taken from the tank in which the temperature during the previous week fluctuated between 18.5° and 19.5° C., and were placed in filtered sea water. The temperature was maintained at 22.6° but occasionally rose for a short time to 23.4°. Continuous kymograph records of shell movement of each of the females were obtained. The first oyster was kept under observation for 5 hours, 22 minutes; the second for 29 hours, 14 minutes; and the last one for 73 hours, 13 minutes. None of the oysters spawned during the experiment but all three shed eggs after sperm was added to the water (22.6°), the latent period being 16, 24, and 15 minutes respectively (Galtsoff, 1932). During the course of the investigations a large number of similar cases were recorded. Undoubtedly some of the oysters which were kept only for 24 hours at a constant temperature might have spawned if the experiments had been continued for a longer period of time. This was, however, impractical and the majority of the experiments had to be completed within one day. The percentage of ripe females which under those conditions were not responsive to temperature stimulation remains therefore undetermined. Yet the fact that within a range from 22.5° to 30° spawning in some of the females can be stimulated by exposing them to a constant temperature, higher than that of their original environment, is well established.

In a second set of experiments carried out in the summer of 1937, the effect of rapid rise in temperature from about 20° C. to 34°–35° C. was studied. After the oyster was placed in the experimental tank the water was quickly heated by an electric Hotpoint immersion heater, which was immediately removed as soon as the thermometer had registered the desired temperature. Then the water was allowed to cool. Usually it required about 20 minutes to bring the temperature from 20° to 34° C. In several instances oysters spawned before 34° had been reached. Out of 43 females used in these experiments, 17 or 39.5 per cent were stimulated to spawn; 24 or 55.8 per cent failed to react to temperature but were induced to spawn by the addition of sperm; and 2 or 4.7 per cent failed to respond to the combined action of both stimuli. The latent periods of the reactions (Table II) i.e., time elapsed between the moment the heat was turned on and the beginning of spawning, varied from 17 to 48 minutes. One can notice that the lowest average latent period occurs at the temperature between 33° and 33.9° C. On account of a small number of observations it is, however, impossible to state whether the observed differences have physiological significance and the temperature between the 33° and 34° is the optimum for stimulation of spawning in the female. At 35° and above, the adductor muscle contracts and the shell remains closed. With the drop of temperature to 34° the adductor relaxes again. No spawning was observed above 35° and oysters exposed for two minutes to temperatures between 35° and 37° and left after that

TABLE II

Latent periods (in minutes) of spawning reactions stimulated by rapid rise of temperature

Temperature		Latent period			Number of observations
Before spawning	During spawning	Maximum	Minimum	Average	
18–21	32.0–32.9	40	22	33	3
18–20.5	33.0–33.9	20	20	20	2
18–22.5	34.0–34.9	48	17	25.5	12

for thirty minutes at 32° to 33° failed to spawn but immediately discharged eggs upon the addition of sperm.

Sudden rise of temperature from 20° to 32°–35° appears to be a more effective stimulant than long-continued exposure to lower constant temperatures. The question arises whether the high temperatures used in these experiments occur in nature and are therefore significant in the propagation of the species. It is true that in northern waters the bottom temperature of the estuaries rarely reaches 27°–28° C. On the other hand, temperatures above 30° often occur in the coastal waters of the southern states. Hopkins has shown (1931) that in June–August the temperature over the oyster beds in Offats Bayou (Texas) remained above 30° and often exceeded 33° C. And further observations in other parts of the Texas coast, made by the Bureau of Fisheries in 1935, confirmed his findings. Furthermore, oysters exposed at low tide to direct sun rays are often heated to a much higher degree than that used in the experiments. For instance, temperatures from 29.2° to 35.8° were recorded by the author in the tissues of oysters exposed during a hot summer day above low water mark on tidal flats of Milford Harbor (Connecticut) while the water temperature in small tidal pools varied from 26.5° to 37.2°. Thus the temperature to which oysters were subjected in the laboratory lies within the thermal range of their normal habitat and the conditions of the experiment cannot be considered abnormal or even unusual.

Stimulation by Sperm

The sperm suspension used in all the experiments was prepared by mincing and squeezing 1 gram of ripe gonad tissue in a small amount of filtered sea water and diluting it up to 100 ml. Amounts varying from 0.03 to 5 ml. of this standard suspension were added to 30 liters of water in which the female oyster was kept. Mechanical stimulation was avoided by emptying the pipette in the vicinity of the stirrer, several inches from the body of the oyster. The water was agitated, aerated, and kept at constant temperature. This technique was followed throughout the investigation with the exception of a few special experiments in which sperm suspension was injected directly into the pallial cavity of the oyster.

It was shown in a previous paper (Galtsoff, 1930) that in a physiologically ripe female live sperm initiates a series of reactions which culminate in ovulation and rhythmical contractions of the adductor muscle. The first noticeable reaction of the oyster consists in one or several sharp contractions of the adductor usually within a few seconds after the addition of sperm (Fig. 1). This can be regarded as an ejection reaction caused by a chemical stimulation of the neural receptors of the tentacles and mantle. The presence or absence of this reaction is not a sign of the readiness of the female to spawn for the spawned-out and unripe oysters react in a similar manner and furthermore identical response can be obtained with any chemical substance to which the neural receptors of the oyster are sensitive. It is certainly not a part of the complex spawning reaction of the female.

The approach of spawning is manifested by the relaxation of the adductor muscle and decrease in the frequency of muscular contractions. The latent period during which these changes occur can be accurately measured from the kymograph records for the beginning of the spawning reaction is unmistakably marked by a series of rapid rhythmical contractions and the maintenance of a definite tonus level (Fig. 1).

The latent period determined in 101 experiments varies from 6 to 38 minutes. The frequency distribution of latency (Fig. 2) plotted for all these experiments, carried in different years and at temperatures ranging from 22.6° to 30.0° C., indicates the predominance of periods lasting from 9 to 12 minutes. In 78 per cent of the observations the latent period is less than 15 minutes. Its average value computed from all the records is 16.2 minutes. The latent period is independent of the concentration of sperm used in stimulating the reaction. This fact becomes apparent by examining Fig. 3 in which the latency indicated by ▲, is plotted against concentration. The present results confirm previous observations of Galtsoff (1930) in which no significant correlation was found between the two variables (Coef. of cor. 0.093 ± 0.106).

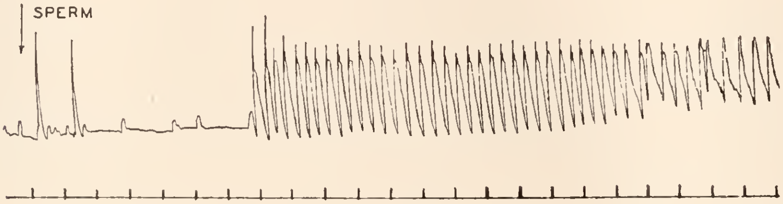

FIG. 1. Stimulation of spawning reaction in female *Ostrea virginica* by sperm. Time interval, 1 minute. Temperature 22.6° C.

There is possibly a slight indirect correlation between the length of the latent period and temperature. Between 25.1° and 30° the peak of the frequency distribution (Fig. 2, blocked-in area) is between 6 and 9 minutes and average value of latent period (22 observations) is only 14.3 minutes as compared with the average of 16.7 minutes and the peak between 9 and 15 minutes at a temperature from 20.1° and 25°

(cross-hatched areas, 79 observations). Both groups, however, cover the entire range of variations.

The next problem deals with the effect of the concentration of sperm on intensity of spawning. If the stimulating effect is due to the direct action of spermatozoa on the receptors of the female one would expect that both the latent period and the intensity of spawning should vary in proportion to the intensity of the stimulus, i.e., concentration of sperm. The duration of spawning reaction can be taken in this case as a measure of the intensity of the response. There is, however, no correlation between the two sets of data, as can be seen from Fig. 3, in which the duration of the reaction is indicated by □. Both the duration of the spawning reaction and its latent period are independent of the intensity of the stimulus.

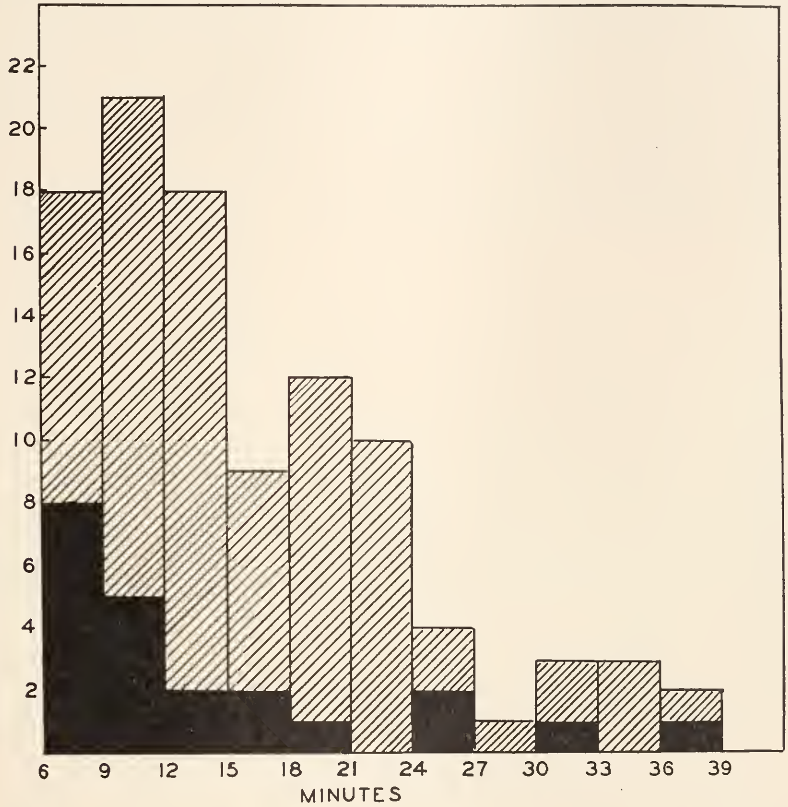

FIG. 2. Frequency distribution of the latent periods of spawning reaction in *Ostrea virginica*. Observations at temperatures between 25.1° and 30° C. are indicated by solid black areas; at 22.5–25.0°, by cross-hatched areas.

The determination of the threshold of sensitivity of the ripe female to the presence of sperm was made by adding gradually increasing amounts of standard sperm suspension to the known volume of water in which the females were kept. The minimum dose of sperm capable of inducing spawning in the female (Fig. 4) was found to be between 0.02 and 0.03 ml. added to 30 liters of water. Counts made by means of a haemocytometer showed that the suspensions used in two experiments contained from 100 to 156 millions of live spermatozoa per 1 ml.

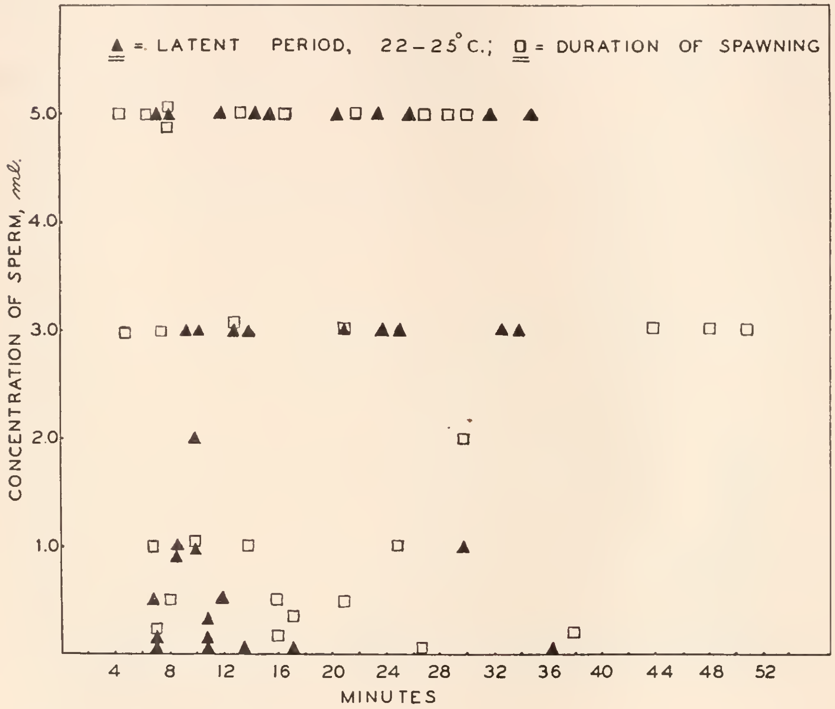

FIG. 3. Independence of latent periods and duration of spawning reaction in *Ostrea virginica* of the concentration of sperm.

or from 100 to 150 (in round figures) per milliliter of water surrounding the oyster. Since in other experiments the sperm suspension was standardized by weight, the exact figures of the number of spermatozoa per ml. probably have no definite significance, especially in view of the fact that the number of other cells present in suspension may vary depending on the state of the gonad development. Furthermore, oysters with greater pumping capacity may filter larger amounts of water and will therefore concentrate in their gills a greater number of spermatozoa than can be accumulated in the same time by a smaller or less efficient mollusk. Present observations indicate, however, that no stimulating effect is to be expected if the number of spermatozoa in the water is below a certain minimum value. An inference can be made that to be effective the sperm must be present in sufficiently high concentration and that the proximity of oysters of both sexes on natural oyster bottoms is essential for the successful propagation of the species.

Immediately after spawning the female which may still contain a large number of ripe eggs becomes non-susceptible to further stimulation by sperm. This refractory period determined in a number of experiments was found to vary in different oysters from two to five days. Histological studies of the tissues of oysters preserved during this non-responsive period fail to reveal any conditions to which this state can be attributed. The ovaries contained a large number of ripe eggs lying free in the follicles and the oviducts appeared to be normal. In a short time the susceptibility is restored and the oyster can again be stimulated to spawn.

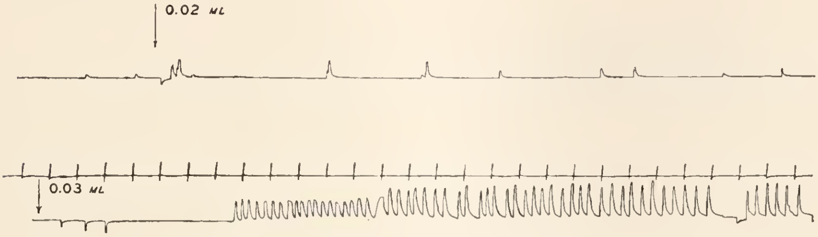

FIG. 4. Threshold concentration of sperm suspension in stimulating spawning reaction in *Ostrea virginica.* Vertical arrows indicate time when 0.02 ml. and 0.03 ml. of suspension were given. Time elapsed between the two tests, 1 hour. Middle line indicates one-minute intervals.

TEMPERATURE REQUIREMENT FOR STIMULATION OF SPAWNING BY SPERM

In the experiments designed to determine the threshold temperature required for stimulation of spawning, ripe females were treated with sperm at 18° C. After the lapse of two hours, during which continuous kymograph records of shell movements were taken, the water was changed, warmed to 20.5° and the oysters given a second dose of the same sperm suspension. In several instances typical spawning reaction took place, indicating that the threshold temperature is between 18.6° and 20.5°. It is of interest to note that at 18° C. some of the females reacted to the presence of sperm by increased muscular contractions. The up and down excursions of the muscle were, however, very small and the reaction was not accompanied by the discharge of eggs. The rhythmicity of these small contractions, not observed under any other conditions, suggested an abortive spawning reaction which failed to reach its full development. Upon raising the temperature to 20.5° C. and adding a second portion of the same sperm suspension, oysters spawned in a typical manner. Similar conditions but differing only in the temperature levels, were observed in *O. gigas* which in one of the experiments at 22.5° responded to the addition of sperm by small rhythmical contractions (Fig. 5, upper line) and spawned one hour later immediately after the temperature was raised to 25° C. (lower line).

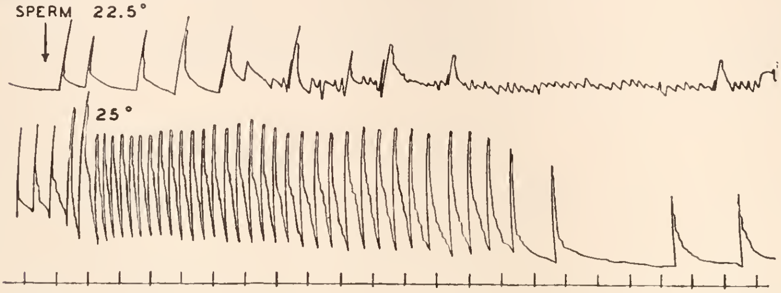

FIG. 5. Effect of temperature on the stimulation of spawning in *Ostrea gigas*. Abortive reaction at 22.5° (upper line); normal reaction at 25° C. Time interval, 1 minute.

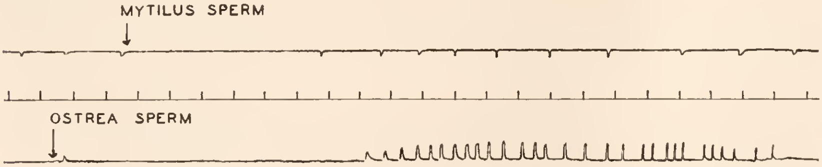

FIG. 6. Specificity of spawning reaction of *Ostrea virginica*. Time interval, 1 minute (middle line).

Can the temperature between 18.6° and 20.5° be regarded as critical for the spawning of *O. virginica?* In papers dealing with oyster spawning, the term "critical" is used in a very unqualified manner, without defining its exact meaning. Experimental evidence points to the fact that temperature around 20° causes no trigger-like effect on the spawning mechanism of the female. A large number of females capable of spawning were found to be unaffected by it and could not even be stimulated at this temperature by sperm. They required warmer water for the initiation of the spawning reaction. Thus we may conclude that the lower limit at which spawning may take place, i.e., between 18.6° and 20.5°, is not a critical temperature in the sense that it initiates ovulation and the discharge of eggs in all physiologically ripe females. Its significance is in the fact that, under laboratory conditions, below this threshold temperature stimulation by sperm is not effective. This conclusion is corroborated by the following observations showing considerable variation in the lower temperature at which spawning can be induced. In the experiments made on June 17, 1936, at 27.5°, sperm failed to provoke the spawning reaction in ripe females which, however, spawned 5 hours later at 33.5° C. Both times the same sperm suspension was used. Spawning was brief with a very light discharge of eggs. In another experiment (Aug. 28, 1934) sperm had no effect on ripe females at 22° C. but stimulation was effected one hour later at 25.6°. An inference from these observations is that sexual responses to chemical stimulation depend on the state of the female organism which may require higher or lower temperature for effectuation of the spawning reaction.[1]

TABLE III

Effect of temperature on the stimulation of spawning by sperm in Ostrea gigas

Date	Temperature	Spawning	Temperature	Spawning
July 17	27.7	–	30.0	+
July 22	22.6	–	30.0	+
July 22	22.6	–	30.0	+
Aug. 8	28.0	–	30.0	+
Aug. 12	28.0	–	30.0	+
July 7*	26.0	–	28.0	+

* Two oysters.

Similar conditions were observed also in *O. gigas*. In the experiments summarized in Table III, each female was given 1 ml. of sperm suspension and kept for one hour at the temperature indicated in column 2. Then the water was warmed to the degree shown in column 4 and a second dose of sperm was administered. Success or failure to spawn is indicated by plus or minus sign. The results clearly show that ripe females non-responsive to sperm at one temperature react very easily to the same suspension at higher temperatures. In more than 30 laboratory experiments carried out with *O. gigas* at Woods Hole no spawning could be induced by sperm at temperatures below 25° C. Yet under natural conditions Elsey (1936) succeeded in stimulating spawning in a large number of oysters of this species at temperatures between 19.4° and 20.2°. Hopkins in a personal communication to the author states that on September 9, 1931, a female *O. gigas* spawned in the laboratory tank in Olympia, Washington, at 16.8° C. The rôle of temperature in controlling spawning in *O. gigas* is not clear, but the observations just quoted indicate a rather wide range under which it may occur. There is a possibility that as the season advances ripe unspawned females become more responsive to stimulation and spawn at lower temperatures than at the beginning of the breeding period. This point requires, however, verification.

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[1] It has been found by Loosanoff that in June, 1938, oysters spawned in Long Island Sound at the temperature of about 17° C. The oyster larvae, however, failed to reach metamorphosis and perished. Some of these oysters taken by the author to the laboratory could not be stimulated to spawn at 20° C. and therefore the conditions which caused their early spawning in the Sound remained undetermined.

Specificity of Spawning Reaction

The question whether the spawning reaction of the female oyster can be stimulated by any other agents besides the sperm of the oyster and an increase in temperature was studied in a series of experiments in which sperm of various mollusks and several hormones were tested. The results were negative. Ripe females were entirely indifferent to the addition of live sperm of *Venus mercenaria*, *Mya arenaria*, *Mytilus edulis*, *Modiolus modiolus* and *Pecten irradians*, but immediately reacted to the addition of oyster sperm (Fig. 6). The injection of thyroidin, theelin, and other hormones which were found to stimulate spawning of the male [2] were entirely ineffective.

So far as chemical stimulation is concerned, the spawning reaction of the ripe female appears to be specific and as present experiments indicate can not be provoked by any other agent except the sperm of an oyster. This specificity is not restricted, however, to the sperm of the same species for it has been found (Galtsoff, 1931) that stimulation of spawning in the female *Ostrea virginica* can be induced by the sperm of *O. gigas*, and vice versa. In both cases typical reaction ensues. Previous experiments (Galtsoff and Smith, 1932) prove that eggs of both species can be cross-fertilized and that the hybrids develop normally. It would be of interest to determine whether spawning in *O. virginica* and *O. gigas* can be provoked by sperm of other species of oysters. Nelson (1931) reports that sperm of *O. angulata* stimulates *O. virginica*, while Galtsoff (1931), when working in Honolulu, was unable to demonstrate any effect of the sperm of *O. commercialis* [3] on *O. virginica* and vice versa. The opportunity to make similar tests with other species has not yet occurred.

The biological significance of the specificity of the spawning reaction is obvious. The oyster discharges its eggs only when the right sperm capable of fertilizing them is present in the water. A non-specific response would result in a waste of the discharged material and eventually lead to the cessation of propagation and the extinction of the species.

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[2] The experiments with males will be fully discussed in a separate paper.

[3] In the above-mentioned paper reference is made to *O. cucullata*. It has been found since that the specimens used in the experiments were *O. commercialis*.

ACTIVE PRINCIPLE OF SPERM SUSPENSION

Attempts to determine the properties of the active principle of sperm suspension were made by testing its solubility in water and in various fat solvents and by ascertaining the effects of temperature and dehydration on its stability.

The first step was to determine whether live sperm is necessary to provoke spawning reaction. The question was answered in the negative by the experiments in which sperm killed by grinding with sand in sea water or in distilled water was used. Sand was purified by repeated washings in water, boiling in concentrated HCl, and washing again in distilled water until the disappearance of the last trace of acid. In killing the sperm the following procedure was used: A piece of ripe testis was ground for 30 minutes with ten times its volume of sand. Then a known quantity of filtered sea water was added, and the mass thoroughly stirred and set aside. As soon as the sand settled the supernatant suspension was pipetted and examined under the microscope. It contained no live sperm. Given to the ripe female kept in water at 23° C. it induced typical spawning, the latent period and the duration of which were within the normal range of the reaction stimulated by live sperm. Eggs discharged by the female were unfertilized and failed to develop during the following twenty-four hours. Identical results were obtained with sperm killed by grinding with sand in distilled water. The tests were repeated several times and always gave identical results which prove that killed sperm does not lose its stimulating power although its fertilizing ability has been destroyed.

The next step consisted in determining whether the active principle is present in the liquid phase of the sperm suspension or is located in the suspended particles. Filtration through tough Watman filter paper Nos. 44 and 52 failed to remove the spermatozoa and the filtrates were as effective in inducing spawning as the unfiltered standard suspension. Complete separation of sperm from the liquid phase has been accomplished by ultrafiltration, using ultrafine Zigmondy collodion membrane capable of retaining Congo Red particles. Samples of clear filtrates obtained in this manner were examined under the microscope and after being found free of suspended material were injected into the pallial cavity of the ripe females. After the lapse of one hour during which no spawning occurred, the residue was collected from the membrane and given to the oyster. As can be seen from Table IV, the filtrate lacked stimulating power, whereas the residue which still contained active sperm, was effective. In all four experiments eggs were fertilized and developed in a normal way.

It is concluded from the results of these experiments that the active principle of sperm suspension is not present in solution but is probably located in the bodies of spermatozoa. Unfortunately, a small number of blood cells and various elements of the connective and epithelial tissues are also present in the suspension. It is, however, unlikely that they are the bearers of the active principle, for it has been determined in a series of experiments that neither the blood nor the tissue juices of the male exert a stimulating effect on the female.

The insolubility of the active principle in the distilled water was demonstrated by ultrafiltering an extract prepared by grinding a piece of testis with sand. In these experiments 1.5 grams of ripe male gonad was minced in 25 ml. of distilled water and ground for 30 minutes in a glass mortar with 5 grams of purified sand. The sand was allowed to settle and a fine suspension was pipetted off and filtered through an ultrafine collodion membrane. The separate tests were performed with two ripe females kept at 20.9° and 21.5° C. Each time a fresh sample of gonad extract was prepared. The filtrates had no effect on the females which, however, reacted in a typical manner to the addition of the residue collected from the membrane. The discharged eggs were not fertilized and failed to develop. Identical results were obtained with sperm extracts in sea water. The experiments demonstrate that the active principle being insoluble in water remains within the cellular fragments of the gonad tissue from which it cannot be separated by this method.

TABLE IV

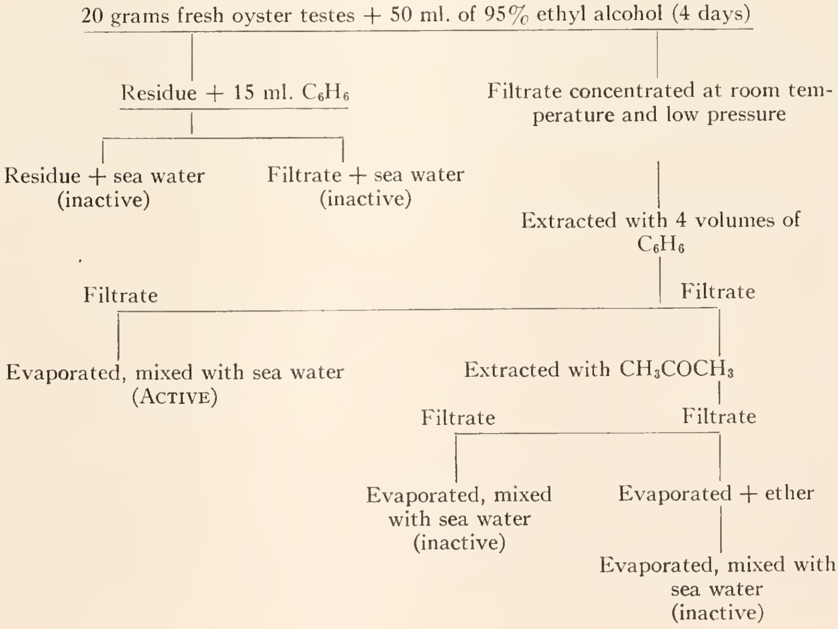
Effect of ultrafiltration on the stimulating power of sperm suspension

Date	Temp.	Membrane	Filtrate	Spawning	Residue	Spawning
	° C.		*ml.*		*ml.*	
June 19.......	23.8	Fine	2.5	–	1.0	+
June 29.......	26.8	Finest	2.5	–	1.0	+
July 21.......	25.0	Ultrafine	10.0	–	5.0	+
July 21.......	27.0	"	10.0	–	5.0	+

EXTRACT OF ACTIVE PRINCIPLE

Since the active principle of sperm suspension was found to be insoluble in water, attempts have been made to extract it in alcohol, ether, benzene, chloroform, acetone, and other fat solvents. Out of many tests, successful results were obtained by employing the method similar to that developed by Koch (1931) and Gallagher and Koch (1929) for the preparation of the male hormone. From 15 to 20 grams of testes of several ripe oysters, after being freed as much as possible from adjacent organs and connective tissue, were finely mashed in a glass mortar and extracted for four days at room temperature with about 50 ml. of 95 per cent ethyl alcohol. The alcoholic solution was filtered and the filtrate evaporated over night in vacuo until an aqueous suspension of lipins was obtained. Since some alcohol still was detectable by smell, about 15 ml. of pure benzene were added and the preparation evaporated almost to the consistency of paste. It was then extracted by repeated shaking with four volumes of benzene until the last benzene portion showed no color. The combined benzene portions were then evaporated to dryness under a vacuum and the extract, mixed with a small amount of filtered and boiled sea water, was injected into the pallial cavity of a ripe female capable of spawning. Out of five tests with this preparation four gave positive results. The reaction induced by the extract did not differ in any way from normal spawning reaction (Fig. 7). The latent periods varied between 10 and 20 minutes. The temperature of the water during the experiments fluctuated between 23.1 and 23.7° C. Attempts to purify the lipin mixture by dissolving it in acetone and separating the inert precipitate were not successful and all the fractions thus obtained had no specific action on females. Various steps of this process and results obtained with different fractions are shown in the following diagram.

Preparation of Sperm Extract


20 grams fresh oyster testes + 50 ml. of 95% ethyl alcohol (4 days)
Residue + 15 ml. C_6H_6
Filtrate concentrated at room temperature and low pressure
Residue + sea water (inactive)
Filtrate + sea water (inactive)
Extracted with 4 volumes of C_6H_6
Filtrate
Filtrate
Evaporated, mixed with sea water (ACTIVE)
Extracted with CH_3COCH_3
Filtrate
Filtrate
Evaporated, mixed with sea water (inactive)
Evaporated + ether
Evaporated, mixed with sea water (inactive)

In all the cases in which the preparation was found to be physiologically inactive, live sperm was given to the female and a positive spawning reaction was obtained, thus proving that the test oyster was capable of spawning. Small yields of active benzene extract precluded the possibility of further chemical analysis.

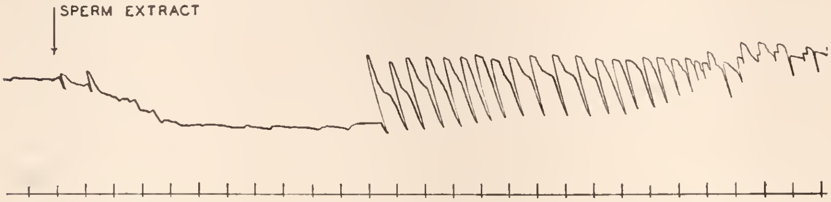


FIG. 7. Stimulation of spawning reaction in *Ostrea virginica* by the sperm extract. Time interval, 1 minute.

INSTABILITY OF THE ACTIVE PRINCIPLE

The active principle is thermolabile and loses its power after a brief exposure to temperatures ranging between 54° and 57° C. This fact was established in a series of experiments summarized in Table V.

TABLE V

Effect of heating on stimulating power of sperm suspension. Second column indicates the temperature at which sperm was tested. Third and fourth columns indicate the temperature used in heating sperm suspension and time of exposure.

Date	Temp.	Heated sperm suspension			Spawning	Normal sperm suspension	Spawning
				Dose			
	° *C.*	° *C.*	*minutes*	*ml.*		*ml.*	
July 8....	25.2	54	15	2	−	2	+
July 9....	23.0	55	15	1	−	1	+
July 10....	23.5	56	15	1	−	1	+
July 11....	23.4	58	15	1	−	1	+
July 13....	23.0	55	15	1	−	1	+
July 13....	23.0	55	15	1	−	1	+

In each of these experiments standard sperm suspension was divided into two portions. One was heated for 15 minutes, counting from the time the desired temperature was reached. The second portion served as control and was administered one, or in some cases, two hours after the treatment with the heated sperm. It is clear from the examination of Table V that 15 minutes at a temperature of 54° and above suffices to destroy the potency of the active principle of sperm suspension.

Microscopic examination showed that all motion in the heated sperm had ceased and some of the spermatozoa were cytolized.

Sperm dried in the oven at 35° C. or at room temperature in vacuo completely lost its stimulating power and had no effect on ripe female oysters which, however, reacted to the addition of live sperm.

Probable Mode of Action of Sperm

Although the mode of action of sperm in stimulating spawning of the female is not known, certain speculation appears to be permissible. Our attention is first focused on the duration of the latent period which, as has been shown above, varies from 6 to 38 minutes. The length of time required for the realization of spawning is significant for it clearly shows the difference between this sexual reaction and an ordinary neuro-muscular response the latent period of which is measured in seconds. Furthermore, the duration of the latent period suggests that sperm may act through the digestive tract or after being absorbed by the gills and mantle of the oyster. The first possibility known to exist in *Platynereis megalops* (Just, 1914) has been investigated in the following manner. A large amount of fine carmine suspension was added to a number of actively feeding adult females. Every three minutes one of them was taken out, dissected and its stomach contents examined for the presence of carmine. It was found that at 22.6° C. a particle suspended in water reaches the digestive tract in about 7 to 12 minutes. It appears to be significant that the time required for the ingestion is about the same as that of the latent period of the spawning reaction. In another set of experiments attempts were made to prevent the sperm from reaching the digestive tract by plugging the mouth with beeswax and plastocele. Yonge (1928) used this method in a study of the absorption of glucose by *O. edulis.* In the present experiments Yonge's technique as well as several modifications of his original method was used without success. In all the cases, the females with plugged mouths spawned upon the addition of sperm. But the dissection of their stomachs revealed the presence of carmine particles which were added to the sperm suspension. This control measure proved the inefficiency of the method in blocking the entrance to the digestive tract. Since carmine particles were not prevented in reaching the mouth and stomach, it is reasonable to expect that sperm also reached the digestive tract. Thus, the experiments failed to throw light on the manner in which sperm acts on females. It is, however, obvious that in stimulating spawning the active principle of sperm does not act directly through the chemical receptors of the tentacles and mantle which, as a rule, respond to chemical or mechanical stimulation within a few seconds.

On the assumption that rhythmical contractions of the adductor may be stimulated by some substance liberated during ovulation and distributed by the organs of circulation, blood of a spawning female was collected and injected in the tissues of another ripe female. In the first set of experiments blood was obtained from females stimulated to spawn by the addition of sperm. In the second series, the use of sperm was avoided and blood was obtained from the females spawning under the influence of temperature. Altogether eight experiments were performed with negative results. The females did not respond to the injection of blood of the spawning females but each of them responded in a typical manner to the addition of sperm. Negative results were also obtained with the blood and tissue juices of the male injected hypodermically or added to the pallial cavity of the female. In preparing the tissue juices the gonads were of course carefully avoided. In all the experiments oysters spawned immediately upon addition of sperm. The results show that of the various tissues of the male organism, testis alone is effective in provoking spawning.

It has been shown in the first part of this investigation (Galtsoff, 1938) that the spawning reaction, once initiated, proceeds in a typical manner irrespective of the nature of the stimulus. Evidence presented in this paper clearly shows that the latent period and duration of spawning reaction are independent of the intensity of the stimulus (degree of temperature or concentration of sperm) and that the reaction proceeds according to the "all-or-none" principle. The rôle of the stimulus apparently consists in setting in motion a chain of reactions which, once started, follow their course and cannot be stopped or inhibited.

Rhythmical contractions of the adductor muscle, typical for spawning reaction, are not caused by the direct action of sperm on the neuroreceptors of the female. This is proven by the fact that spawned-out and unripe females fail to respond to the presence of sperm. On the other hand, the activity of the adductor during spawning cannot be considered as a simple ejection reaction caused by the presence of eggs in the pallial cavity, for the contractions often precede ovulation and sometimes continue after the latter is stopped. The peculiar behavior of the adductor muscle must be attributed to the internal impulses originating in the female organism during spawning. It is a specific sexual reaction of the female. As the breeding season advances, the intensity of the spawning reaction gradually decreases. Completely spent females are as a rule unable to respond to stimulation until the next breeding season. From a biological point of view, spawning reactions of the female oyster present an interesting example of specific physiological adaptation useful to a sedentary organism in providing better dispersal of eggs in the surrounding water and insuring greater chance for their fertilization. The advantages of the reaction in the survival of the species are obvious.

SUMMARY

1. Spawning of the physiologically ripe female *O. virginica* can be induced by exposing it to a constant temperature a few degrees higher than that of its environment or by quickly raising the temperature of the water from about 20° C. to 34–35° C. The second method, which appears to be more effective than the first one, gave positive results in 39.5 per cent of the tests.

2. Sperm of the oyster is a more powerful stimulant of sexual reaction for it easily induces spawning in oysters which do not respond to temperature stimulation. In the laboratory experiments, 55 per cent of the oysters which failed to react to increased temperature immediately responded to the addition of sperm.

3. The latent period of spawning reaction varies from 6 to 38 minutes and is independent of the concentration of sperm. A minimum concentration of sperm of about 100–150 spermatozoa per ml. of sea water is, however, necessary to induce a reaction.

4. The lowest temperature at which spawning in *O. virginica* was induced lay between 18.6° and 20.5° C. This threshold temperature is not, however, a critical temperature in the sense that it induces spawning or makes the stimulation by sperm effective in all physiologically ripe females. The lowest temperature level of the spawning reaction varies in different oysters depending on their responsiveness to stimulation.

5. The spawning reaction of *O. virginica* is specific in the sense that sperm of other genera of mollusks cannot provoke it.

6. The active principle of sperm suspension is thermolabile and insoluble in water. It can be extracted in 95 per cent ethyl alcohol and benzene.

7. The spawning reaction follows the "all-or-none" principle and once started cannot be inhibited or stopped. Rhythmical contractions of the adductor are considered to be due to impulses originating in the female organism during spawning. They are not ejection reactions caused by the presence of eggs in the pallial cavity.

8. The possibility of the action of sperm through absorption by the digestive tract or through the gills is discussed.

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DISTOMUM LASIUM LEIDY, 1891 (SYN. CERCARIAEUM LINTONI MILLER AND NORTHUP, 1926), THE LARVAL STAGE OF ZOÖGONUS RUBELLUS (OLSSON, 1868) (SYN. Z. MIRUS LOOSS, 1901)

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INTRODUCTION

In one of his last publications, Joseph Leidy (1891) described a larval trematode, *Distomum lasium*, from the digestive gland and gonad of *Ilyanassa obsoleta* at Beach Haven, New Jersey. The account, although brief, was sufficiently complete and definitive to permit positive identification of the species. Leidy reported that *I. obsoleta* was very abundant on the mud flats near Beach Haven and that on dissecting a number he "found that one in five or six was infested with the larval distomas enclosed in sporocysts, often in large numbers, embedded in the liver and associated genital gland. The larvae always appeared in the distoma-form and never as a cercaria." He noted the shape and size of the sporocysts and of the tailless larvae, and for the latter he recorded the size of suckers, form of the excretory bladder, presence of spines on cuticula, and stylet in the dorsal wall of the oral sucker.

This species was reported by Linton (1915) from the same host at Woods Hole, Massachusetts. During the summers of 1909 and 1910 he had examined over one thousand specimens of *I. obsoleta* and a record of his dissections is presented. Apparently unaware of Leidy's earlier paper, Linton gave a description of the sporocyst and the cercariaeum, but did not name the species. A comparison of the descriptions of Leidy and Linton shows substantial agreement and leaves no doubt concerning the identity of the parasites collected in New Jersey and Massachusetts.

The account of Linton was confirmed and augmented by Miller and Northup (1926). These authors had likewise overlooked the report of Leidy and they named the larva *Cercariaeum lintoni*. In a study of the seasonal infestation of *Nassa obsoleta* with larval trematodes, they examined 8,875 individuals collected from a limited area in Quamquisset Harbor near Woods Hole, and found *Cercariaeum lintoni* present throughout the year with the greatest infection in December and in June. It increased from 3 per cent in August to 7.5 per cent in December, decreased to 4 per cent at the end of February and began to increase in late May. The incidence of infection in June was not as great as that in December and it declined rapidly during the late summer. Miller and Northup reported on the number of snails from which mature larvae emerged and noted much variation between these figures and the total percentage of infection. They stated that the emergence of mature larvae "continues high from August to December and then apparently is lower until late July and August, at which time it approximates its former values." They discussed the possible effect of temperature and other factors on the seasonal infestation. Their description of the larva was brief and in certain particulars, e.g., the form of the alimentary tract and number of penetration glands, their observations were not correct.

The excretory system of *C. lintoni* was described by Africa (1930) from specimens collected at Cold Spring Harbor, Long Island, New York. He stated, "On account of the numerous refractive granules which obscured the capillary tubes and flame cells especially in the region behind the ventral sucker, great difficulty was experienced in making out these structures. In fact, the exact connection of the two flame cells of the posterior group has not been made out with certainty, but judging from the connections of the anterior apparently homologous groups, they are most likely drained by a common accessory tube just as their anterior neighbors are. It seems quite certain, however, that there are eight flame cells on each side, for in the study of more than a hundred individual specimens there has been no instance when a count of more than eight was made." Africa gave a diagrammatic figure of the excretory system and discussed the mechanism by which the pulsatile contraction of the vesicle is produced. In his figure he copied the incorrect observations of Miller and Northup on the digestive system and penetration glands.

During the summers of 1930 and 1931, Miss C. Ruth Shaw studied *C. lintoni* and carried on experiments to determine the life cycle of the species. Using the methods described by Stunkard (1930*a*), large numbers of normally emerged cercariae were available every day. In her report Shaw (1933) noted that, "Very rarely one was found that had encysted on the side of the dish, but usually they showed gradual decrease in activity unto the point of death." She confirmed Miller and Northup's account of activity and movements of the larvae, and Africa's description of the excretory system. She recognized the two pairs of cephalic glands described by previous workers, and in addition

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six pairs of glands in the region just posterior to the acetabulum. For life history experiments, various marine invertebrates were placed in glass bowls with normally emerged larvae. The cercariae penetrated into and encysted in the body wall of *Nereis virens*, *Hydroides sp.*, *Lumbrinereis hebes*, *Scoloplos robustus*, *Arabella opalina*, and *Bdelloura candida*. The rich blood and oxygen supply in the parapodia of *Nereis* gave especially favorable conditions for the development of the encysted larvae, whereas she found, "In no other host did development occur; on the contrary the larvae died and the cysts became brown and opaque, according to numerous examinations." Shaw also recorded the method of penetration, and the course of development of the metacercariae in the parapodia of *Nereis virens*. Since the organs become larger and more evident as the metacercariae increase in age and size, she was able to distinguish the form of the digestive tract and the relations of the reproductive ducts. Attempts to infect the final host and complete the life history were not successful.

Following the report by Miss Shaw, the experiments were continued during the summer of 1933 in an attempt to complete the life cycle and secure the sexually mature stage of the parasite. Since *Nereis virens* had proved to be a suitable intermediate host, specimens were placed in aquaria with snails from which large numbers of *C. lintoni* were emerging, and the larvae readily encysted in the parapodia and body wall. At the end of two weeks some of the worms had thousands of cysts in the parapodia. Since Palombi (1930) had studied the life history of a similar larva at Naples and found that it developed into *Diphterostomum brusinae*, a fish parasite belonging to the family Zoögonidae, it seemed probable that the adult stage of *C. lintoni* also occurs in the intestine of marine fishes. Accordingly, when the *Nereis* were exposed to infection, specimens of various fishes which frequent the areas where *Nassa* and *Nereis* are abundant were collected and maintained in aquaria. When the fishes had been isolated for three weeks and when the metacercariae had undergone development for the same period in the parapodia of *Nereis*, the infected worms were fed to the fishes. This method permitted development of flukes acquired by the fishes before their capture, and therefore made it possible to distinguish between the experimental infection and any previously acquired one. Fishes were dissected twenty-four hours after feeding, when the recently excysted metacercariae could be easily recognized. By feeding numbers of fishes and killing them at short intervals it was possible to trace the development of the parasite in the final host, and worms with embryos in the uterus were recovered four weeks after the metacercariae were ingested. The metacercariae excysted and young worms persisted for one to three weeks in the intestine of *Paralichthys dentatus* and *Tautoga onitis* but sexually mature specimens were not obtained. Gravid worms were secured from the intestine of *Anguilla chrysypa* and *Opsanus tau*. Attempts to infect *Tautogolabrus adspersus*, *Spheroides maculatus*, *Prionotus carolinus* and *P. strigatus* were unsuccessful. These results were presented in a preliminary report, Stunkard (1933), but since the number of metacercariae fed was enormous and the number of sexually mature worms recovered was small, and since no natural infection was discovered, the experiments have been continued in succeeding years.

Meanwhile Glading (1935) reported the presence of *C. lintoni* in *Ilyanassa obsoleta* from San Francisco Bay, where it had been introduced with the eastern oyster. According to Glading, the sporocysts occur in the liver of the snail, the tailless larvae emerge and encyst in various polychaete annelids; the list included *Capitella sp.*, *Eteone sp.*, *Streblospio sp.*, and *Nereis sp.* These encysted stages he termed "mesocercariae," and he stated that when they were eaten by crabs of the genus *Hemigrapsus*, the mesocercariae excysted, penetrated the intestinal wall and reencysted on the outer surface of the pericardium and in the muscles as metacercariae. When such encysted metacercariae were fed to the gull, *Larus californicus*, they excysted in the duodenum and developed to maturity. Adult worms were recovered near the openings of the ceca and identified as members of the genus *Levinseniella*. Accordingly, the species *C. lintoni* was transferred to the genus *Levinseniella* as *L. lintoni*. Glading declared that the validity of the life cycle as reported was proved by experimental evidence, but the evidence was not presented nor were the control measures stated.

Observations on the life cycle of *Zoögonus mirus* were reported by Timon-David (1933, 1934, 1936). This author described metacercariae, which he identified as larvae of *Z. mirus*, encysted in the muscles of Aristotle's lantern of various sea urchins from the Gulf of Marseille and along the coast of Roussillon. The parasite was abundant in *Paracentrotus lividus* and *Sphaerechinus granularis* and rare in *Arbacia aequituberculata*. Timon-David (1934) gave measurements of the cyst, of the metacercaria, and of its suckers and gonads. Concerning the life cycle he stated, "La contamination expérimentale d'un *Labrus merula* par ingestion d'Oursins parasités est extrêmement difficile à réaliser et présente de très grandes difficultés techniques. Des expériences en cours permettront peut-être une réussite. J'ajoute que j'ai observé fréquemment dans la nature des débris d'Oursins dans le tube digestif de *Labrus merula*." He (1936) reported experiments in which metacercariae were introduced into the pharynx of three fishes, *Blennius gattorugine*. The fishes, sacrificed at the end of 45 days, yielded a dozen sexually immature worms which agreed with the descriptions of *Z. mirus*. Other control specimens of *B. gattorugine* were free of infection.

The metacercariae described by Timon-David were slightly larger than specimens I have taken from *Nereis virens*. The suckers were larger and the gonads were smaller. In my experiments it has been impossible to infect fishes by placing material in the alimentary tract. In every instance food introduced into the pharynx, esophagus or stomach was regurgitated when the fishes were returned to water. Regardless of the feeding experiments of Timon-David, however, the specimens described by him are very similar and may be specifically identical with those I have obtained at Woods Hole.

As stated previously, the work with *C. lintoni* has been continued each summer since 1930 at the Marine Biological Laboratory and it is now possible to present more complete data on the life cycle. Sexually mature worms have been reared in experimental animals and natural infections have been discovered. In abstracts Stunkard (1936*b*, 1936*c*) recorded additional experiments, the results of which confirmed his earlier report. Sufficient material has been assembled to permit a more complete study of the adult which proves to be a member of the genus *Zoögonus*, identified as *Z. rubellus* (Olsson, 1868). The assignment of these specimens to *Z. rubellus* necessitates an inquiry into the status of that species.

The genus *Zoögonus* was erected by Looss (1901) to contain a species described by him as *Z. mirus*. The specific description was based on two specimens found in the terminal part of the intestine of *Labrus merula* collected in the harbor of Trieste. The worms measured 1.55 mm. in length and about 0.45 mm. in greatest width. A conspicuous feature of these flukes was the rudimentary condition of the vitellaria and the absence of shells on the eggs. The miracidia were enclosed in thin, flexible membranes and became ciliated in the uterus.

The following year Odhner (1902) redescribed *Distomum rubellum* Olsson, 1868 and transferred the species to *Zoögonus*. Like Olsson, he found the parasites in *Labrus berggylta* (syn. *L. maculatus*) from the west coast of Sweden, but the examination of twenty fishes at the Zoölogical Station of Kristineberg disclosed only two infections and very few worms were recovered. The specimens were yellowish in color, and situated among the folds of the hind gut. According to Odhner the body length varied from 1.1–1.4 mm. with 0.45 mm. as the greatest width. He distinguished *Z. rubellus* from *Z. mirus* on the size of suckers and length of miracidia.

The description of *Z. mirus* by Looss called attention to the fact that in this species the larvae develop to ciliated miracidia in the uterus and that the eggs are devoid of shells. The latter feature indicated that *Z. mirus* would provide favorable material for the study of gametogenesis, fertilization, cleavage, and early larval development of the digenetic trematodes. Accordingly, a number of investigators undertook the problem and in subsequent years there appeared a series of papers including the classical researches of Goldschmidt (1905), A. and K. E. Schreiner (1908), Grégoire (1909) and Wassermann (1913). In an earlier paper, Goldschmidt (1902) compared specimens collected from *Labrus merula* at Trieste with the descriptions of *Z. mirus* and *Z. rubellus* as given by Looss and Odhner and pointed out that there were no morphological differences. With ample material at his disposal he gave a more complete description of *Z. mirus*, especially of the digestive and reproductive systems. His specimens were undoubtedly specifically identical with those of Looss and the only difference between them and the account of Odhner was in the absence of yellow pigment. He was unwilling to recognize a specific distinction on this feature and pointed out that the dimensions of suckers and miracidia, characters used by Odhner to separate the species, were variable and could not serve as valid specific criteria. In the final report of his researches, Goldschmidt (1905) reaffirmed the opinion expressed in his earlier paper.

In a study of the entozoa of British marine fishes, Nicoll (1909) reported *Zoögonus rubellus* as present abundantly in every specimen of *Anarhichas lupus* from St. Andrews Bay which he examined. The worms were pale yellow in color, 0.75–1 mm. in length, and about one-half as wide as long. Nicoll referred to the descriptions of Looss, Odhner, and Goldschmidt and stated, "My specimens agree best with Goldschmidt's description." It is significant that the worms studied by Nicoll and Odhner came from the same region, whereas Goldschmidt's material was collected in the Mediterranean and presumably was identical with that of Looss. Concerning specific determination, Nicoll expressed the opinion that, "Looss's *Zoögonus mirus* is in all probability identical with Odhner's *Z. rubellus*, . . . at any rate, features sufficient to distinguish them are not at present apparent."

In a systematic review of the family Zoögonidae, Odhner (1911) maintained that the specimens of *Zoögonus* from the Mediterranean and North Sea are specifically distinct. After collecting and comparing material from both locations, he formulated diagnoses for *Z. mirus* and *Z. rubellus.* According to his statement, in *Z. mirus*, "Die länge ausgestreckter Exemplare übersteigt nie 0.6 mm bei einer Breite von 0.18–0.2 mm." In *Z. rubellus* he found, "Länge ausgestreckter Exemplare 0.9–1.2 mm bei einer Breite von etwa 0.25 mm." He stated that the pharynx and miracidia are approximately the same size in the two species but appear to be larger in the smaller specimens of *Z. mirus.*

The Life Cycle of Zoögonus rubellus

During the years 1930–1937 inclusive, over ten thousand specimens of *Nassa obsoleta* from many different localities in the Woods Hole region have been examined for larval trematodes. *Cercariaeum lintoni* has been recovered from every area surveyed, although it was more abundant in certain places than in others. The heaviest infestation was found in snails from Sheep Pen Cove, a sheltered inlet on the northeast side of Nonamesset Island, which opens into Vineyard Sound. The snails on the Sippiwisset side of Buzzards Bay were only slightly infected; sometimes more than five hundred were examined before an infection was encountered. In general, the method employed was to collect 250 snails at a time and isolate them for four days, 10 in a finger bowl, to discover the specimens from which larvae were emerging. Each day the dishes were examined for cercariae and the snails were removed to clean bowls containing fresh sea water. When a finger bowl disclosed an infection, the snails which it contained were isolated separately to find the specimen or specimens which were liberating cercariae. The method obviously does not reveal the total infestation, since not all infected snails are giving off cercariae. Occasionally by crushing and examining snails from which mature larvae were not emerging, one was found which contained sporocysts and immature cercariae. As pointed out in earlier papers (Stunkard, 1930*b*, 1936*a*) such larvae are not infective and so are of little value for experimental purposes, although frequently structural details can be observed best in young cercariae, before the penetration and cystogenous cells are filled with secretory products. By the isolation method an average incidence of infection of about 4 per cent was found for all the snails collected, although much variation was noted. Often two collections from the same locality, taken a few days apart, gave very divergent results. In one collection only three or four infected snails would be found, whereas in the next there might be twenty or thirty. The infected snails were kept in sea water and provided larvae for study and for infection experiments.

The larvae are tailless and unable to swim. They emerge from the snails during both day and night, and temporarily appear in large numbers if the temperature of the water is raised. The cercariae become attached by their posterior ends to the bottom of the dish or to bits of debris on the substratum and constantly extend and retract their bodies. When extended they are four to five times as long as when contracted; the body is narrow and almost round in cross-section. Often it is twisted during extension. The larvae creep about on the bottom by alternately attaching the opposite ends of the body and they are also carried about by currents in the water which move them or the particles to which they adhere. Only rarely do they encyst in the water and ordinarily they die after 24 to 48 hours. The effect of dilution of sea water on the activity and longevity of these cercariae was reported by Stunkard and Shaw (1931). The presence of penetration glands well developed and the failure of the larvae to encyst in sea water indicated clearly the necessity of a second intermediate or transfer host in the life cycle. The structure and habits of the larvae made it manifest from the outset that the second intermediate host must be a bottom-dwelling species, and one which either crawls along or is sedentary with currents of water passing over it. These considerations suggested that this host should be sought among the polychaete annelids. Since the tubicolous species would not be readily infected or readily eaten by the final host, and since *Nereis virens* is prevalent on the sand and mud flats where *Nassa obsoleta* abounds, this polychaete was selected as a likely subject. The experiments with this species were uniformly successful; although the larvae penetrated into and encysted in other annelids, the best results were obtained with *N. virens* (Shaw, 1933). *Nereis* lives in burrows and the movements of the parapodia create a current of water through the tunnel. The current of water draws in small particles and the cercariae which then penetrate the parapodia. The larvae will attack the worms in the open water of a small aquarium, but it appears that they have a much better opportunity of accomplishing infection when the worm is confined within the narrow compass of the burrow. Instances of natural infection are common and frequently the worms were lightly infected when they were collected. The metacercariae were found also in specimens of *N. virens* purchased from the Supply Department of the Marine Biological Laboratory for dissection in the course in general biology at New York University.

Since *N. virens* had proved to be a suitable host for the metacercarial stage, and since the worms are easily maintained in the laboratory, this species was used exclusively for the later infection experiments. Other polychaetes will probably serve as secondary intermediate hosts of the parasite, and Glading (1935) experimentally infected representatives of four different genera. For infection of *N. virens*, large flat dishes half-filled with sand and mud were arranged in series with sea water running through them. Five specimens of *N. obsoleta*, from which larvae were emerging, and ten large specimens of *N. virens* were placed in each dish. At the end of two to four weeks the larvae had encysted in great numbers and each worm contained 1,000–5,000 cysts.

Since infection of *N. obsoleta* with the asexual stages is frequent and widely distributed, it follows that some common and abundant fish harbors the sexual stage. However, many of the common fishes of shallow water were used in experiments to determine whether they would serve as final hosts and provide the sexually mature stage of the parasite. Various species were collected, maintained in the laboratory for three to four weeks, and then fed specimens of *N. virens* which had been exposed for approximately the same time to the larvae from several infected snails. Great difficulty was experienced in maintaining the fishes in small tanks and many of them would not feed. Even though the fishes had been kept in captivity for some time and should have become accustomed to the new surroundings, and although they were probably hungry, it was often very difficult to induce certain of them to eat the *Nereis*. Cunners and *Fundulus* fed readily, but most other species were refractory. Attempts to force-feed the fishes were unsuccessful and worms pushed down their throats were regurgitated. To be sure that a particular fish had eaten an infected worm it was necessary to isolate each fish and watch it until it ate the worm. So worms were suspended by fine thread and allowed to wriggle about in the water near the fish or one was dropped into the aquarium and watched for an hour or two. If at the end of that time it had not been eaten it was picked up and returned to the stock supply. Each day the attempts were repeated. On one occasion when a worm was placed near the head of a flounder, the fish opened its mouth with a yawn-like gape and the *Nereis* swam in the mouth and disappeared, although the fish made no apparent swallowing movement. This fish was dissected four days later and many immature specimens of the parasite were recovered from the intestine. Since the work was done only in the summer months and three or four weeks were required to obtain a large number of experimentally infected *Nereis*, the time left for infection experiments on fish was necessarily short. The list of fishes tested is not large and this portion of the work is not at all complete.

Nicoll found every specimen of *Anarhichas lupus* examined at St. Andrews infected with *Zoögonus*, but this fish is rare and could not be the natural host of the parasite in the Woods Hole region. Since all other previously reported hosts are members of the family Labridae, attempts were made to infect the tautog, *Tautoga onitis*, and the cunner, *Tautogolabrus adspersus*, which are the only members of the family that occur abundantly in the region. Tautogs were infected experimentally but it was difficult to maintain the fishes in our tanks and sexually mature worms were not obtained from them. The cunner is very abundant and from ecological considerations appeared to be the probable natural host. More than fifty cunners were fed infected *Nereis* and although the larvae excysted, the parasites were not recovered from these fishes later than two days after feeding. The sea robins and puffers also either failed to eat the metacercariae or they were lost from the intestine without developing. The parasites persisted for two to three weeks in the intestine of *Paralichthys dentatus* and *Tautoga onitis*, and it is probable that they may reach maturity in these species. The infections lasted throughout the summer in *Anguilla chrysypa* and *Opsanus tau* and gravid specimens were recovered. All stages of development from metacercariae to gravid worms were obtained in experimental infections. Natural infections were discovered in eels and developmental stages identical with those found in experimentally infected fishes were found together with mature specimens. From this material it was possible to identify the specimens with certainty, and their allocation to *Zoögonus rubellus* was announced (Stunkard, 1936*b*, 1936*c*).

The results obtained on fishes in captivity may not entirely parallel those which occur in nature. It is probable that fishes other than eels serve as natural hosts in the Woods Hole region but the feeding experiments gave best results with eels. Eels are common in the areas where the *Nassa* are heavily infected and undoubtedly serve as one of the hosts of the parasite. Examination of eels during successive years has shown a heavier infection in early summer. Our records have been supplemented by data collected by Dr. A. V. Hunninen, who dissected eels during two seasons while working on another problem. Grateful acknowledgment is made to Dr. Hunninen for permission to publish the results of his collection of *Z. rubellus*. Ordinarily the number in a host is one to five, although in one instance 56, and in another 165 worms were removed from a single intestine. Both of these eels were from the Eel Pond. In a series of dissections done in June, 1937, 30 eels were examined. Twenty of them were infected and yielded a total of 339 worms. In another series done in July and August, 1936, the dissection of 100 eels provided only 10 infections and 46 worms. Twenty-seven eels were dissected during the first two weeks of September, 1936, and all were negative. Dr. Hunninen examined 72 eels during the summer of 1937. He found 33 infections and 439 worms, although most of the parasites were collected in June and July.

Since the metacercariae may undergo considerable development in the second intermediate host, it appeared likely that such development was necessary before they could be established in the definitive host, but later observations indicate that the metacercariae are infective very soon if not immediately after encystment. When a toadfish or eel is dissected two days after it has eaten a *Nereis* that had been exposed to infective cercariae for several weeks, one finds a series of young worms. Some of them are only slightly larger than the cercariae and it is apparent that they had only recently encysted and had not undergone development in the worm. It appears therefore that young, recently encysted metacercariae are infective for fishes but that they require a much longer time to reach maturity than metacercariae which have undergone maximal development in *Nereis*. The largest metacercariae require about eight weeks to reach maturity in the final host.

Experimental infection of snails has been produced and, although the time from infection of the snail until the emergence of mature cercariae is unknown, our experiments indicate that more than ten weeks are required. For completion of the life cycle, therefore, about six months are required, although the time varies with the season and inversely as the temperature.

STAGES IN THE LIFE CYCLE OF ZOÖGONUS RUBELLUS

The Adult

Living specimens are very mobile, especially the preacetabular portion; a worm may extend to 1.55 mm. and contract to one-third that length. The width varies inversely as the length and measures from 0.09–0.3 mm. Apart from the development of the reproductive organs and the resulting increase in size, the worms are very little larger than fully grown metacercariae.

Fixed and stained specimens (Fig. 7) measure 0.7–1.2 mm. in length and 0.18–0.28 mm. in width. When alive they may be colorless or tinged a greenish yellow. The yellow material is dispersed in the parenchyma, although frequently the contents of the intestine are more yellow than the tissues and it appears that the color is dependent upon the food of the worm. The cuticula is beset with spines, although they gradually diminish in size and number posteriorly and may be absent on the terminal part of the body. The acetabulum is usually situated about one-fourth of the body length from the anterior end, although this distance varies from one-third to one-sixth. Ordinarily the acetabulum is slightly larger than the oral sucker although both are smaller when contracted than when extended and flattened. In normal worms the acetabulum measures 0.075–0.085 mm. in diameter and in pressed specimens it may expand to a width of 0.1 mm. The oral sucker of mature specimens is 0.07–0.08 mm. in diameter. It is followed by a long prepharynx which is spanned dorsally by the commissure of the central nervous system. The pharynx is spherical, oval to pyriform, usually wider posteriorly, and measures 0.04–0.06 mm. in diameter. Typically it lies a short distance anterior to the acetabulum but the position is very inconstant. The esophagus is long, the bifurcation of the digestive tract ordinarily occurring behind the level of the testes. As noted by Nicoll, the posterior end of the esophagus is often expanded and the ceca arise from the enlargement. The short ceca are either saccate or tubiform and end blindly at or near the ovarian level. Goldschmidt observed that only the fundus of each cecum bears epithelium.

The excretory system of the adult is unchanged from that in the cercarial stage. It is represented by the flame cell formula $2(2 + 2) + (2 + 2)$.

The testes are lateral, situated in the zone immediately posterior to the acetabulum, the testis of the poral side displaced slightly posterior to the other. They are spherical to oval and may be longer in either axis depending on the extension or contraction of the specimen. They measure from 0.032–0.072 mm. in diameter. Vasa efferentia arise from the medial faces of the testes and unite to form a duct, which enters the cirrus sac, where it expands to form a large bipartite, S-shaped seminal vesicle. The vesicle is continued by a narrow duct, surrounded by glandular cells which discharge into it, which communicates with the cirrus. The latter organ is eversible and covered with fine spines, although the spines are often indistinguishable in fixed and stained specimens. The clavate cirrus sac (Fig. 5) is bent laterally, and extends from the region between the testes to the genital pore. The longitudinal muscles of the wall are strongly developed and it may overlie the posterior portion of the acetabulum. The pore, surrounded by conspicuous radial and circular muscles, is lateral, near the level of the posterior margin of the ventral sucker, with the male opening dorsal and anterior to that of the metraterm.

The ovary is spherical to oval, 0.04–0.064 mm. in diameter. It is located between the ends of the digestive ceca, usually slightly lateral in position. The oviduct arises at the median side and turns posteriad where it receives a short duct from the seminal receptacle and where Laurer's canal emerges to pass to the dorsal surface. A small, compact vitelline mass is located beside the ovary and a short duct leads from it to the oötype. The initial portion of the uterus passes forward and coils about. The uterus then passes backward almost to the posterior end of the body, returns to the zone of the digestive ceca, passes backward a second time to the region of the excretory bladder and then runs forward to the genital pore. The loops of the uterus follow a winding course and their position shifts with the extension and retraction of the postacetabular portion of the worm. The portion of the vitelline cells concerned with shell production is absent or inoperative, since the larvae are enclosed in a membranous sac without a shell. When the worms were placed in sea water diluted one-half with distilled water, flattened vacuoles appeared around the larvae and the cilia of the six to ten oldest miracidia beat regularly. The development of the larvae in the uterus was described by Goldschmidt (1905).

The Miracidium

The miracidium (Fig. 8) is ovate, somewhat pointed anteriorly. The body wall consists of four rows of ciliated epidermal cells. The number was not determined precisely and there is apparently some variation in the anterior two rows. As the larvae develop they consist of cell masses which stain deeply but mature miracidia do not stain well and when fully differentiated only a few of the nuclei retain the stain. The anterior tip is very mobile and it may be poked about in exploratory-like movements. There are two large glandular cells which extend more than half of the length of the larva and open at the anterior end. Their contents are finely granular. Immediately behind these glandular cells there is a cluster, usually four to eight, of large cells with deeply staining nuclei. They are undoubtedly the germinal cells which produce the next generation of larvae. There are two flame cells, one situated in the anterior, the other in the posterior portion of the miracidium. Their ducts were not traced to the excretory pores. Living miracidia in their sacs measured 0.1–0.13 mm. by 0.045–0.056 mm. They may become longer and thinner or shorter and thicker. Miracidia in the uterus of fixed and stained worms measure 0.09–0.12 mm. by 0.036–0.045 mm.

When mature worms are removed from the intestine of the eel, the miracidia may be extruded while within their membranous coverings.

The anterior end of the larva emerged first in all specimens observed. Such larvae may escape from their sacs in a short time, and they swim actively in a spiral course. In swimming the body is elongated, and the miracidia swim so fast that it is exceedingly difficult to follow a larva under a binocular microscope. Since the miracidia normally become free-swimming, it is apparent that they meet specimens of *N. obsoleta* and bore into them. Actual penetration was not observed, but the number of swimming larvae was small and the probability of observing one until it found and entered a snail is very remote. It is probable that under natural conditions the miracidia enter the siphon of the snail with the current of water and then pierce the body from some point on the wall of the branchial cavity.

The Sporocyst Generations

The sporocysts which produce cercariae were described by Linton (1915) and Shaw (1933). They are very numerous and are distributed throughout the lymph spaces of the snail, chiefly near the body wall and in the interlobular areas of the digestive gland and gonad. They may be present in different and widely separated locations in the same host and, since they are held together in masses by the connective tissue of the snail and are unable to move about, their distribution indicates that at an earlier period these sporocysts or their progenitor had migrated to the location in which they were found. They are thin-walled oval sacs with a birth pore at one end. At the end opposite the pore a mass of germinal cells and germ balls is followed by progressive development of the larvae until fully formed cercariae are present near the pore. The number of larvae in a sporocyst is variable and apparently depends on its age and condition. In young and vigorous sporocysts there may be 30–40 developing larvae while in older ones there may be only two or three immature cercariae and no germ balls. In snails which had been kept in the laboratory with little food for two or three months and from which cercariae had emerged during all that period, the tissues of the host were emaciated and many of the sporocysts were empty. This observation suggests that the germinal tissues had become exhausted. The cercariae normally leave the sporocysts and complete their development in the lymph spaces of the snail. In the examination of crushed snails, it is frequently impossible to determine whether the free cercariae were liberated from sporocysts in the crushing process or whether they had emerged previously. Study of snails cut in serial sections, however, shows many immature cercariae free in the lymph spaces of the host. Although they may leave the snail by other routes, the examination of many snails, both by dissection and serial section, indicates that the cercariae pass by way of the vascular system to the branchial region, and that when fully mature they break out into the mantle cavity and emerge through the excurrent siphon.

In snails from which cercariae were emerging, all of the sporocysts appear to be producing cercariae. While the germ ball stages of all generations are similar and positive determination may occasionally be impossible, in no case have sporocysts which contain unmistakable daughter sporocysts been found in snails from which cercariae were being liberated. Both daughter sporocysts and cercariae have never been observed in the same sporocyst and it is almost certain that the generations which produce sporocysts are distinct from the one which produces cercariae. Rediae do not occur in the life cycle.

An attempt was made to secure early developmental stages of the parasite in the snail. Fifty small specimens of *N. obsoleta* were collected early in June, 1937, from an area where infection by *C. lintoni* is rare. Forty-eight of them were dissected and proved uninfected; the remaining two were exposed on July 20th to eight miracidia of *Z. rubellus*. No trematode larvae emerged from these snails during the summer and they were killed and fixed entire on September 14th.

EXPLANATION OF PLATES

Abbreviations

cg	gland cell of miracidium	*ic*	intestinal cecum
cs	cirrus sac	*lc*	Laurer's canal
dm	developing miracidium	*mm*	mature miracidium
ev	excretory vesicle	*ov*	ovary
fc	flame cell	*sr*	seminal receptacle
gb	germ ball	*st*	stylet
gc	germ cell	*ts*	testis
gd	ducts of penetration glands	*vt*	vitellaria
gm	degenerating miracidium		

PLATE I

FIG. 1. *Z. mirus*, dorsal view, after Goldschmidt (1902).

FIG. 2. *Z. mirus*, ventral view, after Looss (1901).

FIG. 3. *Z. rubellus*, ventral view, after Odhner (1902).

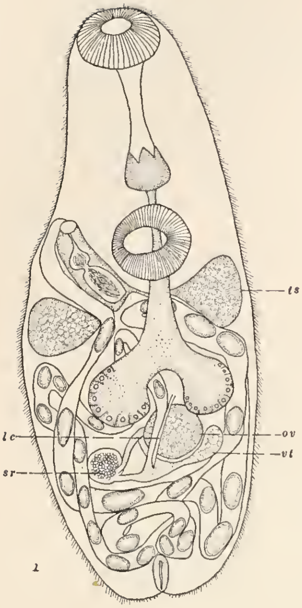
FIG. 4. *Z. rubellus*, ventral view, camera lucida tracing of specimen from toadfish, three weeks experimental infection.

FIG. 5. *Z. rubellus*, free-hand sketch of cirrus sac.

FIG. 6. *Z. rubellus*, ventral view, camera lucida tracing of specimen from eel, seven weeks experimental infection.

FIG. 7. *Z. rubellus*, ventral view, camera lucida tracing of specimen from eel, natural infection.

PLATE I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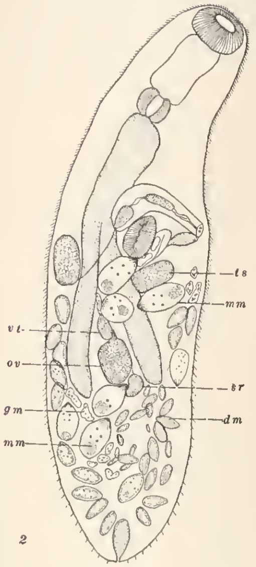
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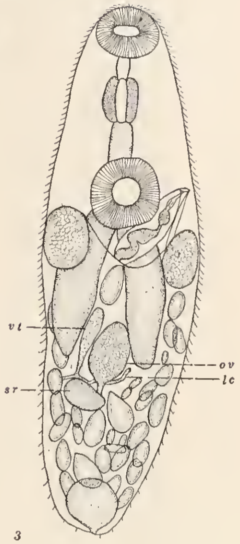

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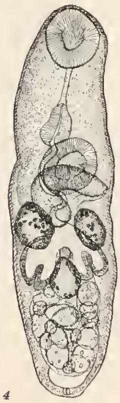

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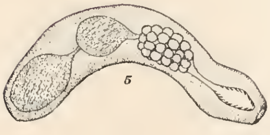

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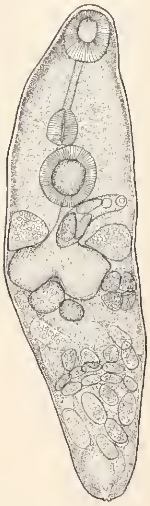

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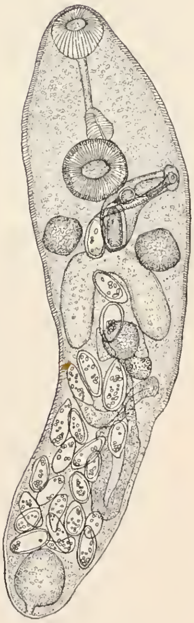

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Later they were cut in serial sections. In both of them the haemocoele spaces contained small sporocysts (Fig. 9), but no other stages were present. The infection is about three times as heavy in one of the snails as in the other. The location of the parasites shows clearly that they migrate by way of the blood sinuses, but in spite of careful search it has been impossible to recognize with certainty the mother sporocyst or sporocysts in which the dispersed generation was produced. The absence of cercaria-producing sporocysts after three months in the laboratory indicates that the snails were not infected before collection and demonstrates that the infection was an experimental one. This conclusion is afforded further support by the uniformity in appearance and stage of development of the parasite in both of the snails. Although they are widely scattered, the sporocysts are very similar in structure. Most of them are solid bodies with aggregations of germinal cells interspersed among the mesenchymal tissues of the parasite. They have irregular shapes and apparently possessed much mobility. These sporocysts frequently contain lacunae or small lumina but the condition of the germinal masses indicates that they are not the definitive generation in which cercariae are developed. In fact, the germinal masses often show secondary aggregations in their interior (Fig. 9) which probably represent another generation. In the germinal tissue, cell multiplication is rapid and mitotic figures are numerous.

The soft parts of *Nassa obsoleta* are usually embedded in the mud and it is likely that the miracidia normally enter the siphon with the incurrent water and from the branchial cavity penetrate into the tissue of the host. Although information concerning the sporocyst generations is yet too fragmentary to permit a final conclusion, a tentative interpretation of the findings in the experimentally infected snails may be made. There is strong probability that each of the miracidia which entered the snails underwent metamorphosis to form a primary sporocyst and that this generation had become unrecognizable after producing a generation of secondary sporocysts which had left the original one. The identification of the sporocysts which were widely

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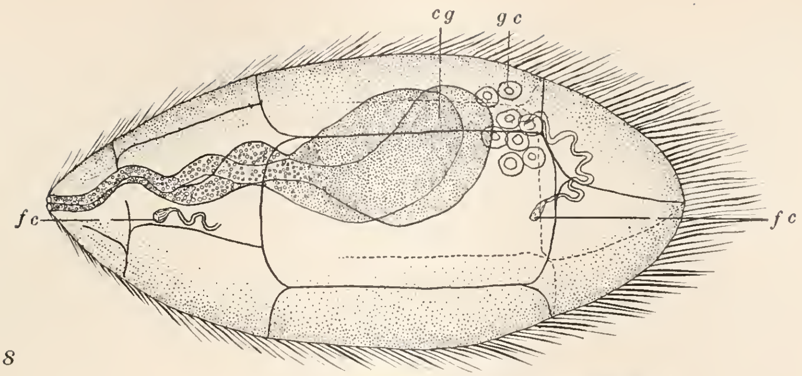
PLATE II

FIG. 8. *Z. rubellus*, free-hand sketch of miracidium.

FIG. 9. *Z. rubellus*, camera lucida tracing of a section of a sporocyst containing germ balls, experimental infection.

FIG. 10. *Z. rubellus*, free-hand sketch of stylet and openings of penetration glands.

FIG. 11. *Z. rubellus*, free-hand sketch of cercaria, dorsal view, stained with neutral red.



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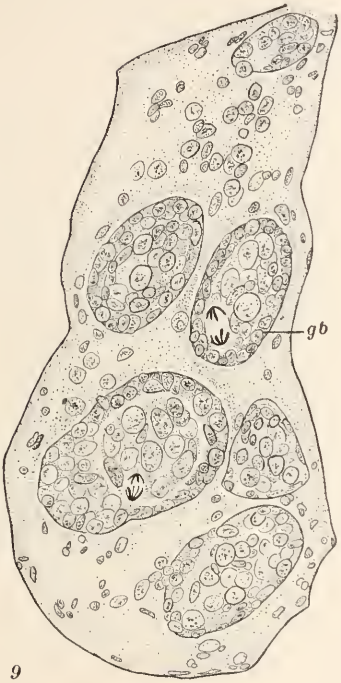

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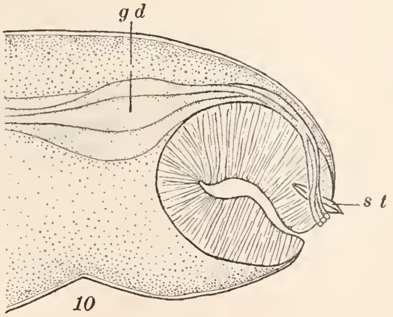

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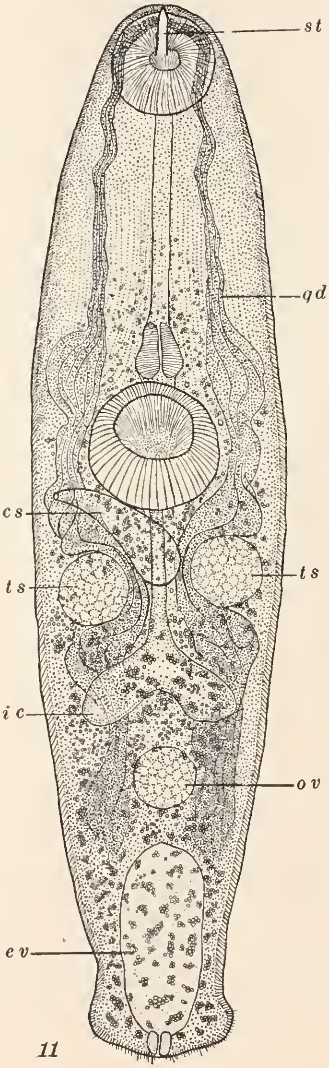
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scattered nine weeks after infection is not an easy matter. It is clear from their number that they are not primary sporocysts. They may be secondary sporocysts, although their number and the time interval, July 20–September 14, which had elapsed since the infection of the snail, suggests that another generation may have intervened. If they belong to the second generation, the primary sporocyst must have produced about 100 daughter sporocysts which were liberated almost simultaneously and in a similar stage of development, with its subsequent retrogression and disappearance. The germ balls in the sporocysts do not have the appearance of developing cercariae, as observed in natural infections, and it is probable that another generation of sporocysts occurs before the production of cercariae.

The Cercaria

Descriptions of the cercaria have been given by Leidy, Linton, Miller and Northup, Africa, Shaw, and Glading. My observations concerning size, shape and movement agree with those of previous authors. The posterior end of the larva (Fig. 11) may be expanded to form a rim, and the contraction of the center of the tip produces a suction by means of which the cercaria becomes attached. The first clear and accurate account of the digestive system was made by Shaw (1933), who corrected the erroneous descriptions of Miller and Northup and of Africa. The study of metacercariae permitted the identification also of the reproductive organs, rudiments of which had been observed by Linton and by Miller and Northup. Linton's observations concerning the testes were confirmed, and the structures tentatively identified by him as the primordia of the ovary and uterus were shown to be those of the copulatory organs and female genital complex respectively.

The description of the excretory system given by Africa was confirmed by Shaw although the details had been worked out previously as noted by Stunkard (1932). The vesicle is saccate and may extend two-fifths of the distance to the acetabulum. From the sides, near the anterior end of the vesicle, collecting ducts pass anterolaterad and then bend mediad, passing forward in a sinuous course below the digestive ceca and median to the testes. On either side in the region between the testes and the acetabulum, the primary collecting duct divides into secondary anterior and posterior branches. The anterior branch continues forward, lateral to the acetabulum, and in front of the sucker it divides into tertiary anterior and posterior branches. The tertiary anterior branch continues forward to the level of the posterior margin of the nerve commissure where it divides to form the capillary tubules to the anterior pair of flame cells. The cells lie equidistant, one in front and the other behind the commissure. The anterior cell is situated ventrally, at the side of the oral sucker, below the ducts of the penetration glands. The capillary which leads to the posterior member of the anterior pair of flame cells may parallel the tertiary duct for a short distance but the cell body is usually lateral in position. The tertiary posterior branch is recurrent and runs parallel with the anterior secondary duct for a short distance. It soon divides into two capillaries, one of which leads to a flame cell situated near the anterior margin of the acetabulum and the other to a flame cell lateral in position near the level of the caudal margin of the sucker. The arrangement of the excretory structures in the posterior half of the body is the counterpart of that in the anterior half. The posterior secondary duct passes backward beside the primary one for a short distance and then divides to form anterior and posterior tertiary branches. The anterior branch is short and divides into two capillaries, one of which leads to a flame cell at or near the level of the testis and the other to a flame cell somewhat more posterior in location. The posterior branch runs backward almost to the level of the vesicle where it divides to form capillaries that supply the caudal pair of flame cells. One of these cells lies in front and the other behind the opening of the collecting duct into the vesicle.

The number and arrangement of the unicellular glands in the cercaria has proved an exceedingly difficult problem. Miller and Northup reported, "One pair of prominent larval gland ducts, with granular contents, on either side of body, mistaken by Linton for excretory vesicles; four large eosinophilic larval glands, staining also with intra-vitam neutral red, but clearly observable only in sections." In their figure two large gland cells were portrayed on each side, behind the acetabulum, with ducts which pass forward to open near the stylet. Concerning the location and nature of the "four large eosinophilic larval glands," the authors made no statement. Miller and Northup's description of the penetration glands was copied by Africa. Shaw stated, "The two pairs of cephalic glands shown in Fig. 1 have been described by previous workers. But in addition, by intra-vitam staining with neutral red, I have been able to recognize six pairs of glands in the region just posterior to the ventral sucker." The outlines of these cells were sketched in the figure by Shaw, although their function was not discussed.

For six seasons I have studied the unicellular glands of this cercaria. The parenchyma is filled with refractive spherules which interfere with observation of the gland cells. Various techniques and many vital dyes have been used in an attempt to differentiate between cystogenous and penetration glands, and to determine the number and position of each type. The application of vital dyes to living specimens has given better results than the staining of fixed specimens. In young cercariae, removed from sporocysts, the gland cells are not recognizable. As the larvae mature the secretion appears in the form of fine granules, but does not stain. The cells are small, difficult to observe, and the two types are morphologically identical. Later, in penetration glands, the granules increase in size and pass into the ducts, where they are stainable. The two pairs of cephalic or penetration glands described by previous authors are merely enlargements in the ducts of glands, whose cell bodies lie farther posteriad.

Instead of two pairs of penetration glands, there are certainly five and probably six pairs of them. The bodies of these cells lie in the region behind the digestive ceca and on either side the ducts intertwine with each other and with the principal excretory tubules to form a bundle which leads forward. From the level of the digestive ceca to that of the oral sucker, the ducts of the penetration glands parallel those of the excretory system and this association increases the difficulty of counting them. The course of the ducts has been noted in the description of the excretory system and is represented in Fig. 11. At the anterior end, the ducts pass over the sucker on either side (Fig. 10) and open ventral to the tip of the stylet.

When the cercariae become active, the contents of the penetration glands pass forward filling the ducts, and the cell bodies are relatively inconspicuous. The cell bodies are lobed, irregular in size, changeable in shape, and often superimposed in such a way that it is impossible to resolve the cell boundaries separating overlapping lobes. The nuclei are seldom clear, although occasionally some of them stand out conspicuously. One cannot be certain, however, that the nuclei of all these cells are visible at the same time and accordingly the number of penetration glands cannot be determined with certitude by counting their nuclei. The ducts of the penetration glands twist about one another and are invisible when empty, so that ordinarily it is not possible to trace a tubule very far. A duct may be much increased in diameter for a short distance and then it may disappear as it passes between other ducts. As the body of the cercaria extends and retracts, the gland ducts are bent and bowed and the secretion accumulates at particular locations; e.g., at the anterior and posterior margins of the oral sucker, acetabulum, and testes. In these regions, expanded portions of the ducts may simulate cell bodies and have undoubtedly misled earlier observers. Occasionally clear spherical areas may present the appearance of nuclei, but their size is inconstant and they are probably vacuoles since they may pass along the ducts and sometimes they disappear or break up into smaller spherules. The secretion is usually clear when extruded and persisting granules soon liquefy.

Cystogenous glands have not been reported previously. These cells are scattered over the body from the level of the oral sucker to the caudal end. Twenty-six of them have been counted in front of the acetabulum. There is a cluster behind the acetabulum and they extend into the region of the penetration glands. At least sixteen are present in the area around the excretory vesicle. The positions and shapes of the cells change with movements of the worm and it has been impossible to trace the ducts of these glands. The cells measure about 0.012 mm., and the nuclei about 0.006 mm. in diameter. By the use of vital dyes it is sometimes possible to distinguish slight differences in form, size, and color between the cytoplasmic inclusions of cystogenous and penetration glands. When treated with dilute neutral red, cystogenous cells may have flaky, reddish granules and penetration glands more spherical and somewhat violet-colored granules. But the form of the inclusions is not constant and, like the staining reaction, probably differs in various stages of the secretory cycle. Similar changes in color reactions of penetration glands in schistosome cercariae were discussed by Stunkard (1930*a*). In *C. lintoni*, the position of certain gland cells may indicate their nature, but in the region of the digestive ceca it is frequently impossible to distinguish between cystogenous and penetration gland cells.

The Metacercaria

The metacercarial stage was first secured experimentally and described by Shaw (1933). Later accounts have added little to her report. In the second intermediate host the cercarial structures are resorbed or lost and development is continued. Since the larvae develop in the annelid, it serves as a true intermediate rather than a mere transfer host, and this fact is probably correlated with a certain degree of specific relationship between host and parasite. Whether the larvae will infect sea urchins at Woods Hole remains to be discovered.

Development in the Final Host

Although the metacercariae excyst in the intestine of several species of fish, mature specimens have been recovered only from the toadfish and the eel. In abnormal hosts the worms are soon lost and even in natural infections the number of parasites recovered is usually small. Natural infections at Woods Hole have been found only in eels, although laboratory experiments indicate that the toadfish may serve as a final host.

The length of time required for the attainment of sexual maturity depends on the degree of development attained by the parasite in the second intermediate host. In a metacercaria which has reached maximal size, the anterior portion of the body and the suckers are almost as large as in the adult condition. The development in the final host therefore consists almost entirely in the maturing of the reproductive organs. The male system develops first and spermatozoa were observed in the seminal vesicle of specimens removed after one week in the intestine of a toadfish. At that time the prostate cells are active and the cirrus is spined. Fertilized ova produce germ balls which increase in size and differentiate into miracidia in the uterus of the worm. As noted previously, there is no shell and the larvae are enclosed by thin, flexible, membranous coverings. Fully formed, ciliated miracidia were obtained seven weeks after fishes had ingested mature metacercariae. If the metacercariae are less well developed, a correspondingly longer time is required to reach maturity. As the worm becomes gravid, the postacetabular region of the body increases in size. Specimens recovered after three weeks and seven weeks in the intestine of the final host are depicted in Figs. 4 and 6 respectively. A fully mature worm from a natural infection is portrayed in Fig. 7.

Discussion

For more than thirty years there has been difference of opinion concerning the validity and specific distinctness of *Zoögonus rubellus* and *Z. mirus*. Goldschmidt (1902, 1905) found the only difference between the two forms to be one of color, and that morphologically they were indistinguishable. The same opinion was expressed by Nicoll (1909). The figures of *Zoögonus* by Looss (1901), Odhner (1902) and Goldschmidt (1902) are reproduced for comparison (Figs. 2, 3, 1). The account of Odhner (1911), maintaining the existence of two distinct species, *Z. rubellus* from the North Sea and *Z. mirus* from the Mediterranean, was based on a comparative study of specimens which he had collected from the two localities. Odhner distinguished the two species on differences in color and size, size of suckers and shape of sucker cavities, location of the acetabulum, length of digestive ceca, position of cirrus sac, and number of eggs in the uterus. The specimens of *Z. mirus* studied by Odhner were small and apparently somewhat contracted. His statement that the length of extended worms never exceeds 0.6 mm. cannot be accepted since the type specimens described by Looss measured 1.55 mm. in length. The size of suckers in *Z.*

mirus as given by Odhner does not agree with the statement of Looss, whose measurements of *Z. mirus* agree with Odhner's figures for *Z. rubellus.* It is to be remembered that Looss had only two specimens and, as noted by Nicoll, the worms were somewhat distorted which accounts for his observation that the acetabulum is slightly smaller than the oral sucker. Furthermore, in soft-bodied worms, which are capable of much extension and contraction and in which one part of the body may be retracted while another is extended, the movements of the animals cause constant variation in the shape and relative position of the organs. It is apparent from Odhner's account that his specimens from the North Sea were more extended than those from the Mediterranean, and this condition may explain the differences noted in the position of the acetabulum and cirrus sac and in the shape of the digestive ceca. The specimens from the Mediterranean were smaller, and the fact that they contained fewer larvae indicates that they were not fully mature. Indeed, the size of the body is largely influenced by the degree of sexual maturity and the worms increase in size as the number of eggs increases. Color is a variable feature and an unsatisfactory criterion for specific determination. Nicoll reported that his specimens from *Anarhichas lupus* were pale yellow, which suggests a condition intermediate between those observed by Odhner. An examination of Odhner's paper and comparison of his account with earlier descriptions left grave doubt concerning the specific distinctness of *Z. rubellus* and *Z. mirus.*

The study of specimens from the eel and toadfish at Woods Hole has provided data for a solution of the problem. Worms recovered at four-day intervals from experimentally infected fishes show a continuous series of developmental stages. Some of the specimens which have developed for seven to eight weeks in the intestine of the final host agree entirely with Odhner's diagnosis of *Z. mirus*, while older infections yielded worms which were more mature and except for the intense yellow color, agree with Odhner's description of *Z. rubellus.* Some of the present specimens were colorless, while others had a faint greenish-yellow color. Since Odhner's diagnosis of *Z. mirus* does not agree with Looss' original description of the species, and since specimens from experimental infections show transition through a series of forms intermediate between *Z. rubellus* and *Z. mirus*, there appears to be no adequate criteria for distinguishing between them.

The work of Palombi (1930) showed that *Cercariaeum crispata* Pelseneer is the larval form of *Diphterostomum brusinae* Stossich, a member of the family Zoögonidae. In that paper Palombi pointed out that *C. lintoni* is very similar to the larva of *D. brusinae* and suggested that it might also be a member of the same family. Palombi found that the life cycle of *D. brusinae* requires about six months for completion and he questioned the explanation offered by Miller and Northup that "semi-annual visitation of Woods Hole by the definitive vertebrate hosts" might account for the two periods of maximal infection of snails in June and December. He found similar high incidence of infection in mollusks in August and February and attributed this fluctuation to the developmental cycle of the parasite. The results of the present study confirm in large part the postulates of Palombi.

The report of Glading (1935) that *C. lintoni* is the larval form of a species of *Levinseniella* may be explained on the assumption that the crabs to which he fed infected polychaete annelids were already infected with metacercariae of the latter genus and that they developed to maturity in the gull, *Larus californicus*. Indeed, Lebour (1911) and more recently Young (1938) have shown that the metacercariae of *Levinseniella* and related forms do occur in crabs.

Discussion of the life history of *Z. rubellus* requires consideration of *Cercariaeum reticulatum*, a species described by Stunkard (1932) from *Nassa reticulata* at Roscoff, France. The original description is brief and incomplete in many respects. This larva is very similar to *C. lintoni* and they are certainly closely related. In fact, review of notes and sketches made at Roscoff indicates that they may be identical. The discovery of the life cycle of *C. lintoni* opens the way for an experimental attack on the problem.

The group *Cercariaeum* Diesing, 1855 has been used to contain a number of little known and doubtful forms. It was restricted by Lühe (1909) to distome larvae without caudal appendages, which develop in rediae or simple sporocysts. Palombi (1930) showed that *Cercariaeum crispata* Pelseneer is the larval stage of *Diphterostomum brusinae*, a member of the family Zoögonidae. Stunkard (1934) reported the life cycle of *Typhlocoelum cymbium*, a member of the family Cyclocoelidae, and showed that the larva is a *Cercariaeum*. In that paper he stated that since members of *Cercariaeum* develop into such widely different adults as *D. brusinae* and *T. cymbium*, it is apparent that the group is an assemblage of unrelated species and has no genetic unity. The discovery of the life history of *Z. rubellus* confirms that opinion.

The larvae of digenetic trematodes have various types of caudal appendages and it is not surprising that in certain of them such structures should be entirely lacking. The term *Cercaria* may be applied with equal propriety to all of these larvae, and the name *Cercariaeum* should be suppressed.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

JULY 5, 1938

Endocrine activity of the placenta in mice. W. H. Newton. (The published paper will appear in an early number of the *Anatomical Record.*)

Evidence for hormonal control of uterine motility by the hypophysis in the rabbit. J. K. W. Ferguson and H. O. Haterius.

By inserting fine bipolar electrodes into the brain of a rabbit it is possible to apply electrical stimulation to the region of the pituitary stalk. When this is done in rabbits two to eighty hours after parturition while recording the motility of one horn of the uterus, an enormous increase in the frequency and amplitude of uterine contractions is observed after a latency of two or three minutes. This effect of electrical stimulation of the stalk is strikingly similar in form and duration to that of 0.3 units of Pitocin injected intravenously. The effect of epinephrine injected intravenously (1 cc. of 1/50,000) is very different, being chiefly an apparent inhibition. Furthermore, the response to epinephrine lasts only a few minutes while the response to Pitocin or electrical stimulation may last for nearly an hour.

The response to electrical stimulation is abolished by destruction of the pituitary stalk and posterior lobe or of the stalk alone. It is not abolished by severing all nervous connections between the brain and the uterus provided the pituitary complex and its connection with the uterus by the blood stream remains intact. After destruction of the stalk the activity of the uterus slowly but progressively diminishes. The evidence establishes to our satisfaction that a hormone capable of stimulating uterine motility can be released from the pituitary *in vivo.* It also suggests that the uterine activity of the parturient animal is directly dependent on the release of a pituitary hormone. Similar experiments on virgin rabbits have given indefinite results.

Experimental studies on the genital system of the male anuran. Roberts Rugh.

By means of micro-injection of a solution (made up of one part India ink and two parts Holtfreter's modification of amphibian Ringer's) into the vas efferens in the direction of the Anuran testis, the distribution of the seminiferous tubules can be made out. If the injection is made in the direction of the kidney, the path of spermatozoa through the kidney to the ureter can be seen. The vas efferens (of which there are 8–12 branching ducts) branches as it enters the kidney, and feeds 4 or more Malpighian corpuscles. Ink can be seen in the Malpighian corpuscle and passing out dorsalward (in the coils of the uriniferous tubules) through the kidney substance and then laterally across the kidney to the ureter (Wolffian or mesonephric duct).

If a hibernating male frog (*Rana pipiens* or *Hyla crucifer*) is injected with the anterior pituitary hormone, and the urinogenital system is studied histologically at intervals of 9–36 hours after treatment, spermatozoa may be traced from their Sertoli cells (in the seminiferous tubules), through the vasa efferentia, into the Malpighian corpuscles, through the uriniferous tubules to the ureter and ultimately to the seminal vesicle—in a manner exactly similar to the course of ink traced by the above-mentioned technique. In *Rana pipiens* about 50 of the 2,000 Malpighian corpuscles have this dual function, and in *Hyla crucifer* about 40 of the 400 are so involved.

This study offers two major conclusions: First, the anterior pituitary hormone acts on the testis of the hibernating frog in a manner similar to its effect on the anuran ovary, i.e., it liberates the gametes. This liberation in the testis may be aided (as it is in the ovary) by muscular contraction of the gonad stroma. Second, the path of spermatozoa is from the seminiferous tubule through the vas efferens to the Malpighian corpuscle, at which point the genital ducts and urinary ducts become one and the same. There is no morphological or histological evidence of any Bidder's canal running along the mesial margin of the kidney, as described for many European Anura.

JULY 12

The rôle of chromogenic bacteria in ciliate growth. G. W. Kidder and C. A. Stuart.

It has been found that there are marked differences in the reactions of the ciliate *Colpoda steini* toward the various species of bacteria normally found in hay infusions. Sixteen types were tested and comparisons made of the ciliate reproduction in suspensions of pure strains in distilled water. The ciliates were sterilized before each test by centrifugation and dilution, and the sterility tested by broth inoculations and agar plate streaks.

It was found that the best growth and reproduction could be obtained in suspensions of *Aerobacter cloacae.* Decreasingly successful were pure suspensions of the following types: *Serratia marcescens* (non-pigmented), *Spirillum rubrum, Vibrio* sp., *Sarcina lutea, Staplococcus aureus, Pseudomonas* (3 species), *Rhodococcus roseus, Flavobacterium* (lemon yellow) and *Staphlococcus albus.* It should be noted that abnormalities occurred when the ciliates were fed on suspensions of *Rhodococcus* and that death rather than the normal encystment followed a short reproductive period in suspensions of *Staphlococcus albus.*

Another group of bacteria failed to support growth entirely. *Flavobacterium* (orange) proved to be non-toxic but entirely rejected as food by the ciliates. Pigmented *Serratia marcescens* caused death in all trophic organisms within 5 to 30 minutes, the red pigment concentrating in the cytoplasm. Ninety per cent of the cysts were likewise killed by the pigment which entered the protoplasm through the cyst wall. *Chromobacterium violaceum* affected the ciliates in a manner similar to the pigmented *Serratia* except that there were no survivals. The water-soluble pigments of the various species of *Pseudomonas* tested and of *Bacillus niger* were instantaneously fatal to all ciliates.

Because of the effects noted above and especially because of the ability of *Colpoda steini* to select particular strains of bacteria suitable as food, it is evident that in a mixed culture there will arise a preponderance of rejected bacteria which will in turn prove detrimental to the ciliates. It is therefore extremely important to sterilize completely all ciliates before attempting any critical observations on nutrition. In addition to the original sterilization, rigid bacteriological techniques are required to prevent future contaminations.

Some effects of temperature on the reproduction of Chilomonas paramecium. Jay A. Smith.

The medium used in the experiments was composed of inorganic salts plus sodium acetate treated to insure sterility at all times. The temperature varied only 0.25° C. at all temperatures, namely 35°, 30°, 27°, 23°, 18°, 14°, and 9° C.

After extended periods of time at various temperatures, the frequency of division became constant. This frequency of division was zero at 9.5° C., but the chilomonads survived for five weeks at this temperature. As the temperature rose, the frequency of division increased to a maximum of about 0.14 divisions per hour between 26° and 30.5° C. Above 30.5° C. the frequency fell until it reached zero at 35° C.

As the chilomonads were exposed to temperatures of 14° C. and 9.5° C. for prolonged periods of time, the temperature and the length of the exposure to the temperature necessary to kill them decreased. This decrease was from 168 hours at 35° (after exposure to 14° C. for 1 week) to 48 hours at 35° C. (after exposure to 14° C. for one week followed by 9.5° C. for 3 and 4 weeks), and from 35° C. to 30° C. The latter temperature became a lethal temperature only after exposure to 14° for one week followed by 9.5° C. for three and four weeks. This decrease was probably due both to a decrease in the frequency of division and to an increase in the death rate.

After prolonged exposure to temperatures of 14° and 9.5° C. the frequency of division at higher viable temperatures was slightly lower than the normal frequency of division at these higher temperatures. This low frequency of division probably had its source in the frequency of division during the first week after transfer from the low temperatures because after extended periods of time at any viable temperature the frequency of division assumes a constant value that is reproducible at that temperature.

During the first 48 hours after transferring the chilomonads from a low viable temperature to other viable temperatures, there was a gradual increase in the frequency of division from the low value at the lower temperature to higher values at the higher temperatures. This indicates that the acclimitization necessary to allow the chilomonads to reproduce at higher temperatures is not an instantaneous acclimitization, but a gradual change, taking 48 hours or so.

Adjustment of the marine Amoeba, Flabellula mira Schaeffer, to changes in the total salt concentration of the outside medium. Dwight L. Hopkins.

This amoeba can be cultured in any concentration from sea water diluted 20 times to sea water concentrated 10 times by evaporation. It never forms contractile vacuoles which are typical for fresh water protozoa. The food vacuoles when extruded from the cell contain large quantities of water as well as fecal material. The rate of elimination of fluid material by means of the vacuoles is inversely proportional to the concentration of the medium and directly proportional to the volume of the amoeba provided that the amoeba has been adjusted to the medium and is active and feeding.

When the concentration of the medium is decreased, the amoeba swells at first and then shrinks to its original volume. During this shrinkage elimination of fluid by the vacuoles does not nearly account for the volume loss. If the concentration of the medium is increased the amoeba shrinks at first and then swells to its original volume. Once its volume has become stable at a given concentration a small increase in concentration will cause shrinkage, indicating an osmotic value for the protoplasm after adjustment only slightly above that of the medium. Thus we may conclude that when sea water is diluted or concentrated this amoeba automatically loses or gains osmotically active substances to or from the medium respectively such that when adjustment is completed the osmotic value of the cytoplasm is but slightly higher than that of the medium and that this is accomplished independently of the action of the vacuoles.

Phenomena of excystment in Colpoda cucullus. C. L. Claff.

Moving pictures showing induced excystment of *Colpoda cucullus* from its protective cyst by the use of laboratory grade distilled water.

July 19

The steady state frequency of the embryonic fish heart at different cyanide concentrations. Kenneth C. Fisher and Richard Ohnell.

When intact embryos of *Fundulus*, salmon or trout are exposed to solutions containing cyanide the heart rate is observed to become constant at a fraction of the normal frequency. There is a maximum reversible inhibition of the frequency which can be produced in this way—it can be obtained usually with N/1,000 or up to N/250 solutions. This maximum effect in the case of *Fundulus* amounts to a lowering of the frequency of the heart to about 27 per cent of the normal; in trout and salmon to about 55 per cent of the normal.

It has been observed both in *Fundulus* and in salmon that the maximum slowing produced by cyanide increases as the temperature rises. These results may be interpreted to mean that there are at least two separate series of chemical reactions involved in the determination of the frequency and that one of them contains as an integral step the Warburg-Keilin oxidase system. Cyanide, by combining with the atmungsferment, stops one of the chains and hence lowers the frequency to the value which can be maintained by the second or other chains. The effect of temperature on the maximum cyanide effect indicates that the temperature coefficient of the cyanide-sensitive chain is greater than that of the remaining series of reactions.

At constant temperature we find in *Fundulus* that as the cyanide concentration is lowered from N/1,000 to N/20,000 its effect decreases to become small or completely lacking. Referring only to that fraction of the frequency which is cyanide-sensitive, the degree of inhibition is related to the cyanide concentration by this equation

$$\frac{\text{uninhibited frequency}}{\text{inhibited frequency}} = K[\mathrm{CN}]^{-1.5}\ (K \text{ at } 23^\circ = 1.5 \times 10^{-6}).$$

This is precisely the relation predicted by the law of mass action if cyanide is combining with and hence inactivating an essential catalyst in a series of reactions which contribute to the determination of the frequency.

These observations fit well with the conception that the frequency of the heart is determined by the velocity of two or more chains of processes which together gradually build up an unstable condition, the sudden dissipation of which, in the pacemaker, constitutes the stimulus to the heart muscle.

Studies on the refractory state resulting from the repeated injections of adrenal extract. Lena A. Lewis, Frank A. Hartman and Kenneth C. McConnell.

It has been reported from this laboratory that the injection of large amounts of adrenal extract into the normal human being and the normal dog causes marked changes in the electrolyte excretion. The effect is differential, the excretion of sodium and chloride being decreased and that of potassium and ammonia increased. The total base excreted remains essentially unchanged. It was further shown that repeated intravenous injections of adrenal extract eventually produce a condition in which there is no response.

Harrop and Thorn, however, failed to observe the development of a refractory state following subcutaneous injections.

Further studies on the conditions producing the refractory state are reported here.

Normal adult male and female dogs, adult female cats and a young immature female pig on a constant diet and regime were studied. The adrenal extracts used were Upjohn Company extract, Wilson Company extract and extracts prepared in this laboratory according to the method of Cartland and Kuizenga.

Subcutaneous injections failed to develop a refractory state, even after many injections over a long period of time (3 dogs used). These findings confirmed the observations of Harrop and Thorn.

Experiments in which a mixture of adrenal extract plus pig serum was injected subcutaneously repeatedly, gave no indication that the refractory state was due to haptene formation.

The refractory state was produced in one dog and one cat by repeated intraperitoneal injections.

Dogs which were refractory to beef adrenal extract, when injected with pig adrenal extract gave a typical electrolyte response. Similarly, dogs refractory to both pig and beef adrenal extracts gave a response to horse or sheep adrenal extract (4 dogs). The refractoriness developed to adrenal extracts is thus species specific.

A young pig failed to develop the refractory state after 8 intravenous injections of beef and 8 of pig adrenal extract. Her age may have been a determining factor in this failure to produce refractoriness.

Due to the ease with which the refractory state is produced by intravenous and intraperitoneal injections it would appear unwise to use these modes for injection of adrenal extracts in the treatment of Addison's disease.

Action potentials of visceral smooth muscles. Emil Bozler.

All-or-none conducted responses can be elicited in strips of visceral muscles by electric stimuli. It has been shown previously that conduction in these responses is muscular and occurs through syncytial connections between the smooth muscle cells. In agreement with this result the action potential during a single conducted response is a wave of negativity differing from that of a nerve only quantitatively by the time factors involved. Monophasic potentials are brief negative variations.

The spontaneous contractions of the uterus and intestine are tetanic contractions, as shown by the observation that there is a burst of impulses during each contraction. Tonic contractions are accompanied by a regular discharge. During a peristaltic wave of the ureter a repetitive discharge of high frequency was observed in the guinea-pig, a slow negative variation in the ureter of other species.

Rhythmical changes in blood flow through muscles. Laurence Irving.

Blood flow was examined in muscles and in other tissues by means of a heated electrical resistance wire type of flow meter which operates on the principle of the hot wire anemometer. In about 25 per cent of the cats under chloralose-urethane anesthesia rhythmical changes in arterial blood pressure were observed amounting to from 10 to 20 mm. in height and in a period of from 4 to 2 per minute.

As the blood pressure rose the flow through muscles diminished and the flow record indicated that the rhythmical changes in pressure originated in changes in the blood vessels of the muscles. Severing the voluntary nerves or the corresponding sympathetic supply changed the muscle flow waves in such a way that flow now passively followed pressure changes. In the brain flow likewise rhythmically and passively followed the pressure changes.

The rhythmical changes in pressure and flow were not closely related to the control of breathing; for if the breathing were altered by (1) the inhalation of CO_2 or a deficient O_2 mixture, or (2) by apnea caused by inflation of the lungs, or (3) by vagotomy the flow rhythm was not significantly changed.

The rhythmical change in flow was not closely related to arterial pressure for it persisted practically unchanged (1) when the blood pressure was depressed by the Valsalva effect or (2) when blood pressure was raised by carotid sinus stimulation from clamping the carotids. The important respiratory afferents in the vagus, depressor and carotid sinus nerves were not essential, for the rhythm appeared after their denervation.

These observations indicate that the rhythmical changes in pressure originate largely in the vascular bed of the muscles and that they are activated over the sympathetic pathway. The rhythm must originate in a vasomotor center which is relatively independent of the control of respiration. The nature of the rhythmical activity in the periphery indicates the type of rhythmical activity within the center and suggests how the activity of the center may be examined by the behavior of the peripheral system it controls.

JULY 26

Skin transplants between embryos of different breeds of fowl. B. H. Willier and Mary E. Rawles.

Small pieces (1.0 × 0.5 mm. or less) of skin ectoderm, with some adhering mesenchyme, stripped from the head of 72-hour chick embryos and transplanted to the right wing bud base of hosts of the same age but different breeds, produce large areas of donor-colored down feathers covering the entire wing and often adjacent parts of breast, back and thigh. Skin ectoderm from other regions of the embryo grafted to the head, wing, leg or tail bud gives the same result but with less spread on the head. After hatching, the down of these areas is gradually replaced by contour feathers which have the donor color and pattern but the form, rate of growth and tract arrangement characteristic of corresponding regions in host controls.

Host-graft combinations of White Leghorn, Rhode Island Red, Barred Plymouth Rock, F_1 hybrid (Barred ♀ × R. I. Red ♂), Black and Buff Minorca, White Silkie bantam, White Wyandotte and White Plymouth Rock have been tested. Areas of donor-colored feathers have been obtained in all combinations except those in which White Leghorn was donor to Barred Rock, F_1 hybrid and R. I. Red. Combinations of the various white breeds have always produced white feathers.

That the feathers of the graft area are structurally derived from host epidermis is demonstrated in two ways: (1) Structurally normal contour feathers (lack of barbicels is a Silkie character) of donor color develop when Silkie ectoderm is grafted to Black Minorca hosts. (2) Implants of limb bud mesoderm alone from Barred Plymouth Rock embryos produce in White Leghorn hosts donor-colored feather areas similar in all respects to those obtained from skin ectoderm grafts (Watterson, unpublished).

From these results and an histological study showing that the implanted skin ectoderm does not replace host ectoderm in the graft area, the interpretation is reached that the feathers of the donor-colored feather area are produced conjointly by (*a*) follicles composed of host epidermal cells and of (*b*) chromatophores or diffusible substances originating from the implant. In some way the color produced is controlled by the donor cells of the implant.

Induction by cauterization in the amphibian egg. Arthur Cohen.

Tissues and extracts of tissues of almost all groups of animals when implanted into amphibian gastrulae may produce secondary induced structures. Barth obtained inductions by implanting digitonin and suggested the latter injures the adjacent cells which then liberate the inducing substances. If this were so it should be possible to obtain inductions without implanting any substance but simply by injuring the inner layer of the presumptive non-neural ectoderm. This was done by inserting a micro-cautery needle into the blastocoele of young gastrulae of *Rana pipiens*. As a result, protuberances have appeared over the injured area.

Two criteria can be used to identify material of ectodermal origin in these embryos and early tadpoles; the presence of numerous pigment granules and the reduction or absence of yolk granules. The induced structures consist of ectodermal thickenings which give evidence of having been folded in from the surface as is the case with medullary plates; of tubes possessing well-defined lumina, possibly neural in nature and resembling the nerve tube at the extreme tip of the tail of a normal embryo; and in one case, of striated muscle which appears to have been formed directly from the ectoderm. In all the induced structures the presence of many pigment granules and the absence of yolk granules point clearly to derivation from the ectoderm. Moreover, many of the induced structures remain either in continuity or in contact with the ectoderm. The ectodermal origin of the induced muscle is further emphasized by comparison with the normal myotome muscle of the same tadpole. In the latter case the muscle cells are still well filled with yolk but only sparsely pigmented.

Apparently the cells which are injured by the cautery needle liberate the inducing substances which then act on the surrounding ectoderm and so influence the course of its differentiation as to produce structures which do not normally arise from this portion of the ectoderm.

The innervation of transplanted limbs in chick embryos. Viktor Hamburger.

Wing and hind limb primordia of chick embryos (60–70 hours of incubation) were transplanted onto the flank or into the coelom of host embryos of the same stages. Only transplants which were located dorsally were found to be innervated. In these cases, nerves from the adjacent region of the spinal cord of the host entered the transplant and formed in it a more or less complete and typical pattern. Trunk nerves alone or in combination with limb nerves furnished the nerve supply of the transplant. It is to be concluded that the normal nerve pattern is determined by factors intrinsic in the limb, and is independent of the source of origin and of the quantity of ingrowing fibers.

Striking illustrations of the non-specificity of nerves were found in cases in which, e.g., a single branch of a brachial nerve was capable of forming an almost complete brachial pattern in a transplanted wing; or in another case in which a leg transplant replaced the host wing, and the brachial nerves of the host formed a typical leg pattern in it.

The ingrowing nerves responded to the increased demand by hyperplastic growth. This hyperplasia, however, is definitely limited; the transplants were never filled to capacity with nerve fibers. Apparently, two antagonistic mechanisms are at work: one, a growth stimulus exerted by the transplant; the other a growth-limiting factor intrinsic in the nervous system which latter sets the upper limit to the hyperplasia. The actual size of a nerve is the resultant of these two components.

The situation parallels closely that found in experiments on amphibian embryos.

The effect of mechanical stress on cartilage differentiated in vitro. Paul Weiss.

When pre-cartilage of the chick embryo is allowed to develop in tissue culture under constant mechanical stress, the resulting cartilage shows typical trajectorial patterns. The cells are oriented and arranged along lines of maximum strain. Thus, the functional adaptation of cartilage observed in the organism can be reproduced and analyzed in vitro.

August 2

Some recent studies on barnacles. J. P. Visscher.

We have worked with the larvae of some forty species of barnacle with reference to factors determining attachment. Some are attracted by certain environmental factors and others repelled. Most barnacle larvae are positive to light when first hatched, but all littoral forms with which we have worked are known to be negative to light at the time of attachment. Not only light but pressure and oxygen concentration have their rôle in determining the behavior of many forms. As illustration of this fact, we have found a remarkably constant distribution of the four most common species around Woods Hole. Another series of barnacles is found only in association with crabs, and at Beaufort, North Carolina, a barnacle is found growing only in association with the Gorgonean corals.

All of these cases indicate selective attraction with reference to the cyprid larvae.

In conclusion we may note a few specific items as follows:

1. That there are six stages in the nauplius life of the barnacle—six nauplean moults.
2. That the distribution of barnacles is determined by the behavior of larvae and subsequently by the food supply.
3. That certain chemical substances are attractive and others repellent to the cyprid stage.
4. That the adults of several species of barnacles survive in fresh water but that their larvae do not.
5. That there is no evidence of distribution of barnacles from one port to another by virtue of the attachment on bottoms of ships.
6. That it is the larval life of barnacles which controls the distribution of these forms.

Observations on some of the lower Turbellaria of the eastern United States. E. Ruffin Jones, Jr.

A study of the *Acoela*, *Rhabdocoela* and *Alloeocoela* from various parts of Virginia and North Carolina as well as from Woods Hole has been undertaken. Even though the work is still in its early stages, six species hitherto unreported from this country have already been identified, including under the order *Rhabdocoela: Macrostomum stylopencillum*, a new species from North Carolina; *Omalostomum schultzei* (Claparède) 1863 from Woods Hole; *Solenopharynx flavidus* Graff 1882 from Woods Hole and Virginia; *Sopharynx oculatus* (Pereyaslawa) 1892 from Woods Hole; *Polycystis mamertina* (Graff) 1874 from Woods Hole; and under the Alloeocoela, *Enterostomula graffi* (Beauchamp) 1913 from North Carolina. In the last-named form the presence of the ductus spermaticus and vagina externa which were observed in the American representatives of the species necessitates its removal from the genus *Monoophorum* and its inclusion in the genus *Enterostomula*. The excretory system in this species consists of a pair of protonephridial tubules near the mid-ventral line and extending from the region of the cephalic ganglia to the urinary sinus, a collecting chamber which opens into the penis sheath. The tubules have a type of construction which is believed to be peculiar to this species.

Some problems of distribution and variation in the Hawaiian tree snail Achatinella. d'A. A. Welch.

Large collections of the tree snail *Achatinella* from the Island of Oahu have been collected by various people and given to the Bishop Museum in Honolulu. Dr. C. M. Cooke of that Museum has encouraged collectors to collect from highly restricted localities of not over 100–300 square yards in extent. For the past six years much time has been devoted to plotting these localities on the U. S. G. S. Top. maps of the Island of Oahu, also to collecting new material, checking as far as possible all localities and studying the organized material.

Two species have been dealt with so far. One of these, on which the work has not been completed, is in the Koolau Range and is named *A. apexfulva*. The study of the other species is now in press and the paper is entitled "The Distribution and Variation of Achatinella mustelina in the Waianae Mountains." *A. apexfulva* is used to illustrate the color variation of a species on only two ridges in the Koolau Range. On one ridge a series of six different color patterns occurs in definite areas at successively higher elevations. The color ranges from a streaked blue gray color pattern at the lowest elevation to non-streaked pink and white patterns at higher elevation areas. The next main ridge north of this ridge, just described, has a totally different set of six color patterns, which also occur in distinct areas on the ridge at different elevations.

At present there seems to be a zonal arrangement of color patterns in the Koolau Range as well as a valley to valley variation. These color zones are present on all the ridges in the Koolau Range from which there is material and seem to occur at definite distances from the main division ridge. In the case of *A. apexfulva*, they do not appear to be correlated with any specific elevation. Streaked forms occur at the lower elevation zones while non-streaked forms occur in higher elevation zones. Although a change in color pattern in different sectors on a ridge seems to be a fairly orderly thing, the occurrence of different colors in various regions in all the lower areas of the Koolau Range as a whole appears to be a random affair. In the higher areas within two miles of the backbone there appears to be a tendency to produce white color patterns.

All this applies only to *A. apexfulva*. Another species will show an entirely different altitudinal and valley to valley variation. There are, of course, exceptions to this rule, and possibly all these ideas will be changed when the study of *A. apexfulva* is completed.

The problem of size variation with altitude was studied in *A. mustelina*. Some 15,176 shells were collected from about 582 localities. Each lot of shells from each locality was measured to the nearest millimeter and the mean length obtained for each locality. It was found that a correlation existed between shell size and the amount of annual precipitation in the locality. Shells tended to decrease in size with increase of moisture conditions. This pertains only to *A. mustelina*, for in another species the reverse is true. No size difference was noted between shells collected ten or more years ago from a definitely known restricted locality and shells collected from the same place within recent years.

August 16

Studies on fragments of centrifuged Nereis eggs. Donald Paul Costello.

When the unfertilized eggs of *Nereis limbata* are centrifuged at about 66,000 times gravity for 60 to 80 minutes, the interior protoplasm becomes stratified and the eggs are elongated into a dumb-bell form. Due to their shape, the eggs orient in the centrifuge with the polar axis perpendicular to the axis of centrifuging. To obtain egg fragments, the dumb-bells, after removal from the centrifuge, were cut by hand with microneedles. Three types of cuts were made: "upper," across the neck of the dumb-bell; "lower," across the lower edge of the yolk zone; and "intermediate," through the yolk zone. The centripetal fragment contains the germinal vesicle, and its cytoplasmic constituents differ from those of the centrifugal fragment.

Upon insemination, either fragment, or both, may be activated. While activated centripetal fragments usually cleave, the centrifugal fragments never cleave. The centripetal fragments produced by the "upper" cut rarely develop into larvae bearing prototrochal cilia although they may attain a sufficiently advanced multicellular condition. Ciliated larvae develop frequently from the "lower" centripetal fragments, less frequently from the "intermediate" centripetal fragments. These experiments indicate that some substance in the vicinity of the equator of the strongly centrifuged *Nereis* egg is essential for the development of a larva bearing prototrochal cilia. Whether this material has been displaced into this position by the centrifugal force, or whether it has retained its relationship to the polar axis despite the centrifuging, is a problem which remains to be solved.

Calcium and magnesium in relation to longevity of egg cells. Victor Schechter.

In continuation of the work of last summer on salt effects upon the longevity of unfertilized *Mactra* egg cells, high magnesium concentrations have been consistently effective in prolonging the life of the eggs and also in retaining the spherical form of the egg. The action of low calcium has not been as clear. This may be due to the fact that smaller animals of Nonamesset Flats were used currently and during the early part of the summer, whereas much larger ones coming from colder Cape Cod waters were experimented on the latter part of last summer. In view of these facts, and inasmuch as magnesium and calcium are in many fundamental ways antagonistic, the persistence of the high magnesium effect makes it possible to regard the somewhat inconsistent calcium results, not as contradictory, but as indicating a different level of initial egg condition. These results corroborate experiments performed with the *Arbacia* egg and already published. In view of the delay in breakdown of the germinal vesicle of *Mactra* in the experimental solutions, the taking up of calcium by the nucleus is postulated as an important factor in aging. Previously reported experiments on change in osmotic resistance of unfertilized *Arbacia* egg cells, with age, may also be interpreted as due to an uptake of calcium.

Some properties of living chromosomes. John B. Buck and Robert D. Boche.

A method was developed for studying salivary gland chromosomes in the unequivocally living condition. It consists in flattening the living larva between slide and coverglass and observing and measuring the nuclei and chromosomes directly through the body wall under high magnification. For quantitative work *Sciara* proved especially favorable because the nuclei of the salivary gland are spherical, uniform in size, and regularly arranged.

Study of the salivary glands of four genera of Diptera showed that in the normal living condition the chromosomes fill, or nearly fill the nucleus. Nuclear volume thus equals chromosome volume.

Asphyxiation causes the salivary chromosomes to shrink into a spherical clump, the diameter of which serves as an accurate measure of the decrease in volume. Nuclear volume as a whole remains constant, so that the exchange of fluid is entirely intranuclear. The shrinkage of the chromosomes is reversible up to a maximum of about 65 per cent loss in volume.

Injection of hypotonic and hypertonic Ringer solutions into the larva causes reversible swelling (at least 20 per cent) and shrinking (at least 35 per cent), respectively, of both nuclei and chromosomes.

Mechanical pressure on the cytoplasm causes shrinkage of the chromosomes in the affected cells.

In distinguishing between "living" and "dead" salivary chromosomes it is instructive to draw an analogy to certain features of protein chemistry. As long as the chromosomes are able to take in and give out fluid they are comparable to native proteins, but the loss of this capacity, which is associated with the death of the organism, resembles the irreversible loss of solubility attendant on protein denaturation.

Some cytological studies on virus infected cells. Alfred M. Lucas.

A study of nuclear autolysis has been undertaken in fox and guinea-pigs following infection respectively with fox encephalitis and submaxillary gland disease. The intranuclear inclusion bodies of fox encephalitis are Feulgen-positive. When they appear in the non-nucleolate cells of the anterior medullary velum, which received particular attention in this investigation, the first reaction by the normal components of the nucleus is a pulling together of scattered chromatin granules and movement of them toward the margin. As the process proceeds the oxychromatin separates from the basichromatin and migrates in the opposite direction, namely, toward the inclusion body to which it becomes applied. Later the oxychromatin merges with the inclusion body. The basichromatin completely marginates and forms a thin shell which subsequently breaks up into small granules which aggregate into larger masses during nucleoclasis. Ultimately the nuclear membrane shrinks against the inclusion body, a reaction typical of autolysis.

Submaxillary gland virus produces intranuclear inclusions in monocytes and submaxillary gland duct cells. The inclusion bodies are Feulgen-negative in early stages but become somewhat positive later. In the monocytes the chromatin marginates but does not subsequently coalesce into clumps. The fate of the monocytes is not clear but practically all have disappeared by ten days, at which time early stages in inclusion formation may be found in duct cell nuclei. Only in the duct cells are successive steps of inclusion formation approximately synchronized. Margination process takes place during the first three or four days and this is followed by reverse movement of chromatin toward the inclusion body. The particles are small at first but later aggregate to the condition found in stock animals carrying the disease. This occurs ten days to fourteen days after the inclusions first appear. The cytoplasmic inclusions appear first when the nuclear process is about half completed.

A microdissection study of amphibian chromosomes. W. R. Duryee.

Synaptic chromosomes in the germinal vesicles of ovarian eggs of the salamander, *Triturus pyrrhogaster*, appear to be the largest yet observed. At one stage two pair are over 500 μ long in the relaxed condition. During the years of growth synaptic pairs are shown by microdissection to be fused with one another at the chiasmata.

Toward the end of ovarian development chromosomes become much shorter, consisting of transparent gelatinous cylinders in which denser less elastic segments are imbedded. Consequently in this condition vertebrate chromosomes are comparable in structure to those of the dipteran salivary gland.

However, amphibian chromosomes go through a protracted growth period (in anurans lasting over a year) during which each nodal segment gives off elastic granular side branches (av. 30 μ) in the form of loops. Since these loops are sloughed off from the chromonema cylinder, not being resorbed, both under normal and experimental conditions, we have a concrete example of material being synthesized under the direct influence of each chromomere. Such new material is added to the cytoplasm at the time of germinal vesicle breakdown, if not before.

It has been found that the relative tensile strength of the chromonemata drops from an average increase of 364 per cent in Ca-free Ringer to about 102 per cent when $CaCl_2$ 0.001 molar is present. Under the latter condition chromosomes become more brittle, and tend to come apart at the chiasmata.

AUGUST 23

The relation of the magnesium ion to ultraviolet stimulation in the Nereis egg. Karl Wilbur.

Earlier experiments have shown that the germinal vesicle of the *Nereis* egg, which normally remains intact when the egg is shed into sea water, breaks down when treated with a variety of stimulating agents such as ultra-violet radiation and isotonic solutions of NaCl or KCl. The breakdown is apparently dependent upon calcium, for if this ion is first removed from the egg cortex with sodium citrate these stimulating agents are ineffective.

The magnesium ion inhibits germinal vesicle breakdown produced by ultraviolet stimulation. This was demonstrated by immersing eggs in mixtures of sea

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water and isotonic $MgCl_2$ and exposing them to the unfiltered radiation from a mercury arc lamp. The relation of the concentration of magnesium to effectiveness of stimulation was examined quantitatively.

The inhibitory action of magnesium may be overcome by increasing the irradiation time. Prolonged exposures in sea water give a lower percentage breakdown than more moderate irradiation. Nuclear breakdown with ultra-violet stimulation is not immediate and can be completely inhibited by the addition of isotonic $MgCl_2$ mixtures 3 to 5 seconds following irradiation in sea water.

The inhibitory effect of a sea water-$MgCl_2$ mixture is reduced by the addition of isotonic $CaCl_2$. Enough $CaCl_2$ was added so that the Mg-Ca ratio was made to equal the ratio of these ions in sea water.

Eggs in magnesium-free sea water show a greater percentage of germinal vesicle breakdown with ultra-violet stimulation than eggs in sea water containing magnesium. A few of the non-irradiated eggs in magnesium-free sea water at a pH slightly below 6.0 may undergo spontaneous breakdown, and in a more alkaline solution many of the eggs show spontaneous breakdown, polar body formation and an irregular type of division.

The results indicate that the response of the *Nereis* egg to stimulation is similar to other types of protoplasmic response in its relation to the magnesium ion.

Cytological effects of x-rays on grasshopper embryos. E. Eleanor Carothers.

A study of *Melanoplus differentialis* embryos which had received a single dose of x-rays in early pre-diapause leads to the following observations and conclusions concerning the immediate and latent effects of radiation. The first effect is to speed all cells which are in late prophase or metaphase at the time of x-raying through to anaphase. An apparent inhibition follows since cells which would divide normally during the time of the lag have been rushed through. All cells in late prophase, metaphase or anaphase at the time of radiation die at anaphase with pycnotic nuclei. It is thus purely a matter of chance which cells will be eliminated by this means; except that the cells of regions where certain anlaga are being formed are more active mitotically at particular times with consequently greater injury to those regions, or that cells which are fully differentiated functionally and have ceased to divide (serosa and yolk cells) are protected. Many of the cells with pycnotic nuclei are engulfed by adjacent tissue cells. Two days after radiation metaphases show fragmented and lagging chromosomes but there is no new pycnosis. A second type of injury, characterized by liquefaction of the cytoplasm, is now evident. Many, if not all, of the cytolyzing cells contain ingested pycnotic nuclei. Within five days extensive cytolytic lesions appear. This cytolysis may be due to direct injury of the cytoplasm by the x-rays or to a toxic effect from the phagocytized necrotic cells. Mitosis seems to be stimulated, perhaps due to the liberation of intercellular hormones. Loofbourow ('37) and others have shown that injured cells may yield substances which cause rapid proliferation of uninjured cells. Yolk cells phagocytize injured cells and remove them from the vicinity of the embryo. Growth ensues in many of the embryos with most of the cells now apparently normal. Shortly before diapause and in early post-diapause the nuclei of groups of cells become pycnotic, due probably to lack of necessary cellular associations. Finally, the embryos die, due to lack of organization rather than to absence of differentiation, since all types of tissue may be present.

Quantitative studies on the effect of x-rays on mammalian cells, and on the mode of x-ray action. J. Furth.

The effect of x-rays on malignant blood cells in mice can be studied quantitatively. The number of cells can be estimated (*a*) by injecting a counted cell suspension in diminishing amounts into closely related inbred mice and (*b*) by determining the length of life of mice after inoculation, this being inversely related to the number of living cells injected. Malignant lymphocytes are highly susceptible to x-rays: 200r kills all but about 1 of 100 and 400r, 1 of 1,000 x-rayed cells. Approximately 800r is fatal to all cells. The malignant myelocytes studied are slightly less susceptible to x-rays than the lymphocytes and the monocytes are very resistant. The few cells that escaped destruction behaved like unirradiated cells. The lethal dose of x-ray is approximately the same *in vitro* for leukocytes concentrated in Tyrode solution as *in vivo* for the same number of leukocytes scattered throughout the body. This observation suggests that x-rays affect cells directly. Experiments with parabiotic twins made in association with Drs. A. A. Brower and O. B. Furth, in which one animal was irradiated and the other shielded, support this opinion. Tissue culture studies indicate that death produced by x-ray is delayed by several days or weeks during which the cells migrate actively and probably multiply.

The effect of x-rays on Arbacia punctulata sperm. Paul S. Henshaw.

Delay in the onset of the first cleavage in *Arbacia* eggs may be brought about by subjecting either or both of the gametes to x-rays. One distinct difference occurs, however, depending on whether the sperm or eggs are irradiated. In the case of the eggs recovery has been found to take place both during treatment and during the interval between the end of treatment and the moment of insemination. In the case of the sperm, on the other hand, no recovery takes place during either interval. Thus a much clearer picture of how the delay is produced as a function of the dose of radiation may be obtained by treating the sperm. Ten experiments have been accumulated which are consistent in indicating that the amount of delay produced is directly proportional to the logarithm of the amount of radiation applied.

Further, it has been found that when the dose of radiation is sufficiently large, multipolar cleavage is produced in a large percentage of the eggs. This happens irrespective of whether sperm or eggs are treated but to a larger extent usually when sperm are exposed. Examination of serial sections of the treated material showed that the multipolar condition was not due to polyspermy and that the first manifestation of it was the formation of accessory asters which was followed by multiple spindles and the multipolar cleavage condition. Since these changes may be produced equally often or even oftener when sperm are treated, and since sperm are composed mainly of nuclear material, the evidence leads to the presumption that the multiplicity effects are brought about by changes produced in the nucleus.

Whether delay in cell division and multipolar cleavage are both due to the same initial change produced by the radiation is not clear. Yet, if the inference just mentioned should apply—namely, that multipolar cleavage is due to nuclear changes produced by the radiation—the two reactions would have this in common, for it has been shown in previous work with nucleated and non-nucleated egg fragments that the delay in cleavage results from nuclear changes.

Recovery of Arbacia eggs from high intensity x-ray effects. T. N. White.

Recovery following x-ray treatment at about 500 roentgen/min. has been discussed by P. S. Henshaw (*Amer. Jour. Roent. and Radium Therapy*, Vol. XXVII, p., 890, 1932). Results of similar experiments with x-ray output of about 7,600 roentgen/min. were shown in comparison, and the principal features of recovery under the new conditions were pointed out.

General Scientific Meeting

August 30

Isolation of salivary gland nuclei. A. M. Melland.

Salivary glands of dipteran larvae, chiefly species of *Chironomus*, with nuclei up to 100μ in diameter, were used. As the chromosomes practically fill the nucleus, whole nuclei may be collected for analysis of chromosome material.

Glands were dissected out either in isotonic Ringer's or in the body fluid of the larva; treated as follows; and the nuclei dissected out with fine steel needles.

Unfixed Glands.—The most satisfactory method for obtaining nuclei which, when dissected, will react osmotically to various salt solutions is that of dissecting the glands on an ice-cooled stage after being in .25 per cent dried egg albumen solution for 2–3 hours at 5° C. The nuclei then come out easily and are not too sticky.

Fixed Glands.—In the dissection of impregnated and tanned material the criteria used were the ease of dissection and the resemblance of the dissected to the living nuclei. Glands in isotonic Ringer's were left over the osmium vapour from a 2 per cent solution for 24 hours at 5° C. and the nuclei then dissected out in distilled water. These nuclei do not show any shrinkage of the chromosomes. Glands in 10 per cent formalin for 24 hours at 14° or for 36–60 hours at 8° yielded nuclei which were dissectible in 10 per cent formalin: 50–80 nuclei being dissected per hour.

Cytological studies on the living thyroid of the salamander. Glenn H. Algire.[1]

A method is described for the transillumination of the thyroid gland of the living salamander, with the normal blood supply intact, making it possible to observe and take photographs with the highest oil immersion systems of the compound microscope. The illuminating system differs from that of the conventional microscope in having the two top condenser lenses replaced by a straight quartz rod, through which rays of light are transmitted to its tapered tip by internal reflection. The rod projects through a hole in the animal dish and is sealed against leaks. The operation isolates the thyroid and its vessels from fascial connection with neighboring structures. The gland is brought directly over the tip of the rod by means of Harne micromanipulators. Artificial respiration is accomplished by passing in air through a cannula inserted into the tip of the lung. Specially constructed immersion caps permit the use of the oil immersion objectives for observations of the gland which is immersed in Ringer's solution. The apparatus makes it possible to study, in the living intact animal, the cytological changes which occur in the thyroid cells in response to injections of thyreoactivator. In this manner it has been confirmed that the structures observed in fixed and stained sections are not artifacts, but exist in the living cell. These structures, as described by Uhlenhuth, are: the plasma granule, the colloid droplet, intracellular vacuoles, and intercellular spaces.

Micro-structural changes in muscle fibers after caffeine. Ralph H. Cheney.

The gastrocnemius muscle of *Rana pipiens* Schreber which has been used by the author for a series (1931–37) of physiological studies on caffeine and nicotine effects, was immersed in caffeine-in-Ringer. The concentrations and time intervals employed were represented by 0.03 per cent for 10 minutes and 30 minutes respectively; 0.06 per cent for 30 minutes; 0.12 per cent for 10 seconds; 0.50 per cent for 2 seconds; and 1 per cent for 30 minutes. The preparations were fixed in Bouin's and stained with Heidenhain's iron haematoxylin.

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[1] From the Department of Anatomy, University of Maryland Medical School.

Loss of myofibrillar tension results in a "wavy" appearance, indicating a folding of the Q and J band material without loss of alignment of the cross striations. This phenomenon occurs in normal muscle when excised. Depending upon the concentration and time of immersion, the myofibrillae pass through a definite order of microphysical changes. The chronological stages may be described histologically as (1) *incomplete* or *partial clot* formation, in which the regular cross striation pattern is replaced by local irregular zones in which marked shortening of myofibrillar substance has occurred; and (2) *retraction cap* appearance, involving the rupture of the majority or all interfibrillar Z membranes and the redistribution and "congealing" of Q and J band substances with the formation of Zenker's Waxy Degeneration areas. Retraction caps are abundant in muscles in the state of caffeine contracture.

The sequences of microstructural changes induced by caffeine are similar to (or identical with) the action shown in fixed preparations of striated muscle by Nageotte ('37) to occur in response to extreme faradic current and to chloroform. The stages of alterations in the myofibrillae after caffeine immersion correspond also to the observations of Speidel ('38) on the living muscle fiber after bruising, cutting, electrical stimulation, alcohol, and non-isotonic salt and sugar solutions.

The suggestion presents itself that the sequence of ultimate physical changes in the shortening process of the myofibrillae is a constant factor and largely independent of the nature of the preceding chemical reactions in the sarcoplasm.

Some features of contraction nodes and retraction clots as observed in single fibers of cardiac and skeletal muscle of both vertebrates and invertebrates. Carl C. Speidel.

An abnormal variety of muscle fiber contraction is featured by the formation of a sharply localized node of contraction which comprises relatively few (10–20) sarcomeres. Such a node may form and travel along a fiber to the end, or it may form and disappear without traveling. Successive nodes may travel along a fiber in the same direction, several being present at one time. Two nodes may travel toward one another in the same fiber, collide and disappear. A node may form and divide, each of the two halves then traveling along the fiber in opposite directions. A node may, or may not, succeed in passing a retraction clot in the fiber.

The phenomenon of muscle clotting after injury is characterized by the transformation of cross-striated material into waxy zones of coagulation (Zenker's degeneration). The most conspicuous variety of retraction clot is accompanied by rupture of myofibrils on one or both sides of the clot. A less conspicuous incipient retraction clot may form, however, without rupture of myofibrils. This is a soft thin clot waxy in appearance at first. It may be quickly replaced (within a few minutes or even a few seconds), however, by a cloudy zone. This zone is without cross striae but is still capable of further shortening and clotting. Between these two extremes many intermediate retraction clots occur. Retraction clots vary greatly in number of myofibrils involved. They may be thick or thin, and may be placed across the fiber transversely, irregularly, or in pyramidal, zig-zag, or terraced fashion. Intercalated discs in the heart probably arise as thin retraction clots.

This study is based chiefly on arthropods (*Limulus* and *Palaemonetes*), amphibians (*Rana*, *Hyla* and *Pseudacris*), and mammals (*Mus*).

Illustrative cinéphotomicrographs have been obtained (see abstract of motion picture, p. 359).

The heteropycnosis of sex chromosomes and its interpretation in terms of spiral structure. Michael J. D. White.

There are two kinds of heteropycnosis which may be called negative and positive heteropycnosis. In the X-chromosome of the Acrididae both types are met with, the chromosome reversing its behavior during the course of spermatogenesis. Both positively and negatively heteropycnotic chromosomes have a spiral structure at metaphase. The number of gyres in the spiral per unit length is inversely proportional to the thickness of the chromosome, so that it is greater in negatively heteropycnotic chromosomes than in positively heteropycnotic ones. Probably all metaphase chromosomes are compact spirals in which the gyres are in contact with one another, so that there is no space between them and only a "virtual" space in the axis of the coil. The spiral structure is not apparent, however, until the coils have been relaxed somewhat, so that there is a space between them. The direction of coiling (right- or left-handed) is apparently at random, since in the X-chromosomes of long-horned grasshoppers both kinds are met with in approximately equal numbers in the same individual.

Mitochondria and other inclusions in the ciliate Tillina canalifera. John P. Turner.

Studies on the living protoplasm and of 4μ sections of *T. canalifera* made according to the methods of Champey-Kull, Benda, Regaud, Mann-Kopsch, Ludford, Kolatchev, Hirschler, etc., and *intra vitam* staining have revealed:

(1) Endoplasmic granules $\frac{1}{2}$ to $\frac{2}{3}$ μ in diameter which respond to the mitochondrial techniques, except that they do not stain clearly with Janus green. Many of these granules are elongated into rods and dumb-bells $1\frac{1}{2}$ μ long. Champey-Kull and Benda preparations show a faint clear area around each granule as though it were contained in a vacuole.

(2) Similar granules in the cortex which are more irregular in shape and which take a delayed neutral red stain.

(3) Rods up to 4 μ long and $\frac{1}{2}$ μ thick visible in the living endoplasm as conspicuous, highly refractive bodies. These have an osmiophilic cortex and an osmiophobic center.

(4) Minute osmiophilic granules in the endoplasm which are usually seen collected around an osmiophobic globule so as to form a cortex about it. Forms intermediate between numbers 3 and 4 are also found.

(5) Neutral red-stainable granules in the endoplasm which are extremely minute at first, but which appear to grow in size as the preparation ages. These are frequently concentrated around the cytopharynx and the forming food vacuole.

(6) Fat droplets of irregular size in the endoplasm which stain brilliantly with Sudan III.

Cytoplasmic inclusions and matrix of the Arbacia egg. Robert Chambers.

A striking example of the property of protoplasm to undergo sudden and reversible sol-gel transformation occurs in the light fragments produced by centrifuging sea-urchin eggs. The heavy fragment, containing the bulk of the cytoplasmic inclusions, can be readily churned by pushing with a blunt microneedle. On the other hand, the light fragments, although highly liquid, tend to resist being churned by becoming immediately converted, upon manipulation, into plastic solids. Slight compression causes such distorted, solid bodies to revert to liquid spheres.

The cell inclusions to which particular attention has been given are (1) the fat globules of the so-called oil-cap of centrifuged eggs and (2) the pigment vacuoles or so-called pigment granules.

The fat globules are almost at the limit of visibility, being considerably smaller than one micron in diameter. Each fat globule is enveloped by material which becomes coagulated into a single mass on exposure to heat (70° C.) or to formalin. Gently compressing the brittle mass with a needle causes the included oil to exude and to coalesce into a relatively large drop of oil. The oil-cap is agglutinated by $CaCl_2$ and dispersed by NaCl or KCl.

The pigment vacuoles are about two micra or less in diameter. They are globular and easily deformable. On puncturing with the tip of a fine microneedle, the enveloping membrane disappears, releasing the pigment which diffuses into the cytoplasmic matrix. The same phenomenon occurs when the eggs are exposed to heat and during cytolysis induced by mechanical injury of the eggs. Fat non-solvent, coagulating agents maintain them intact. It is noteworthy that the pigment vacuoles, like many plant vacuoles, are more acid than the surrounding cytoplasm and resemble the plant vacuole in their avidity for neutral red.

The Devaux effect at oil-protoplasm interfaces. M. J. Kopac.

Devaux [*C. R. Acad. Sci.*, **202**: 1957, 1936] reported that minute folds or wrinkles are produced by a monolayer of protein covering an oil-water interface. Such wrinkling is the result of a spontaneous increase in interfacial area and indicates zero interfacial tension. Langmuir and Waugh [*Jour. Gen. Physiol.*, **21**: 745, 1938] named this phenomenon the Devaux effect. Oil-drops in the presence of excess protein also may spontaneously increase their surface area as shown by wrinkling. With low protein concentrations, the interfacial tension, although considerably reduced, remains positive.

The results of testing the Devaux effect on cellular systems are as follows: Oil-drops in contact with protoplasm show no Devaux effect, but a pronounced wrinkling quickly appears after the cell is cytolyzed. Oil-drops in small, incipient cytolytic zones become slightly wrinkled, the maximum effect occurring after extensive cytolysis. Striking results occur when the oil is introduced just prior to cytolysis, the change to zero interfacial tension requiring less than 15 seconds.

There is a significant difference in the effect produced on oil-drops introduced before or after cytolysis. Moreover, the time at which an oil-drop is introduced into a cell is also an important factor. Oil-drops in contact with protoplasm for 5 minutes will not wrinkle after cytolysis, while drops brought in contact from one-half minute before to one-quarter minute after cytolysis quickly indicate a zero interfacial tension. Oil-drops injected from $\frac{1}{2}$ to $1\frac{1}{2}$ minutes later show only a slight wrinkling.

The study of the Devaux effect at oil-protoplasm interfaces suggests two aspects of protoplasmic structure. The first and more obvious one is that proteins do not accumulate on experimentally introduced oil interfaces while the protoplasm is intact. Apparently the proteins are bound in the living cell and released only on cytolysis. The second and less obvious conclusion is that the protoplasmic proteins are essentially unstable, high molecular weight complexes. This point of view makes it possible to show how the Devaux effect is obtained at the instant of cytolysis. The adsorption of a complex aggregate which later dissociates produces the same effect as a high initial concentration of low molecular weight protein. On the other hand, if the large molecules dissociate before they are adsorbed, the initial concentration is, in effect, low. The adsorption of proteins on oil-drops introduced after cytolysis has been demonstrated [Kopac, this number].

Further studies on the permeability of the erythrocyte to ammonium salts. M. H. Jacobs and A. K. Parpart.

By means of the photoelectric method of Parpart (*Jour. Cell. Comp. Physiol.*, **7**, 153, 1935), it has been shown that volume changes of beef erythrocytes caused by the penetration of $(NH_4)_2H$ Citrate are almost negligibly slow. Advantage has been taken of this fact to repeat on the erythrocyte experiments of the sort reported by Jacobs and Stewart (*Jour. Cell. Comp. Physiol.*, **7**, 351, 1936) for the *Arbacia* egg in which an unequal distribution of a penetrating ammonium salt between a cell and its surroundings was brought about by the presence in the external solution of a non-penetrating ammonium salt. The addition of a small amount of concentrated ammonium acetate, valerate, salicylate, etc., causes swelling of erythrocytes in originally isotonic solutions of $(NH_4)_2H$ Citrate but not of NaCl. For reasons discussed in the earlier paper, ammonium borate shows a different behavior. Because of the permeability of the erythrocyte to the Cl ion, the addition of a small amount of saturated NaCl also causes swelling of cells suspended in isotonic ammonium citrate solutions, even though the osmotic pressure of the surrounding medium is thereby increased. This effect is greater when the solution is first made less acid by the addition of NH_4OH. When a small amount of NH_4 salicylate is added to erythrocytes in M/6 NH_4Cl the time of hemolysis may either be decreased or greatly increased according to the time of addition. The volume changes of the cells under these conditions suggest that the NH_4 salicylate has a swelling effect of its own similar to that mentioned above but that at the same time it greatly decreases the permeability of the cell to the Cl ion. No corresponding decrease in permeability to glycerol or thiourea or to ammonium salts which enter the cell by a non-ionic mechanism has been found. Ammonium benzoate has a very similar effect with the erythrocytes of many mammals, but only a slight permeability-decreasing one with those of the rat.

Permeability and the lipoid content of the erythrocyte. A. J. Dziemian and A. K. Parpart.

Stroma were obtained quantitatively from the erythrocytes of the sting ray, smooth dogfish, and rabbit by repeated washings with distilled water and hypertonic sodium chloride. The lipids were extracted from samples of intact erythrocytes of the above species by both the refluxing and cold alcohol-ether methods. The same was done for stroma from equivalent amounts of cells. Aliquots of the above were extracted with petroleum ether and the total lipid content was determined by the gasometric technique. The cold cell, refluxed stroma, and cold stroma extracts showed identical amounts of lipid, but the refluxed cell extract, in most cases, had a greater quantity, possibly due to breakdown products of hemoglobin. It appears that the cold extraction gives a truer picture of the erythrocyte lipids than does the reflux extraction.

Cold extracts were made from beef, rat, rabbit, cat, opossum, smooth dogfish, and mackerel erythrocytes. Fish cells had two to three times more lipid per unit area than did mammalian cells. The mammalian red cells of the species examined had almost identical amounts of lipid per unit area, except in the case of the beef cells, which contained a slightly higher quantity.

While the results, considering the rat and beef erythrocytes, appear to agree with Höber's theories on the penetration of lipid-soluble substances, the difference in lipid content between the two species does not seem to be great enough to account for differences in permeability.

The relation of the shell to anaerobic metabolism in Venus mercenaria. Louis-Paul Dugal and Laurence Irving.

During survival of *Venus mercenaria* when kept out of water the CO_2 content of the mantle cavity fluid increases without much decrease in pH. The shell is visibly eroded under the center of the mantle. The calcium of the fluid increases more than the CO_2, indicating the formation of other acids. The small change in pH indicates that the other acid is neutralized and buffered, and glass electrode titration shows that its pK corresponds with that of lactic acid. The volatile weak acid in the fluid as determined by titration agrees with the CO_2 determined by the Van Slyke method. The sum of the CO_2 and other acid is equivalent to the calcium. From these relations it is evident that the CO_2 gained by the fluid is derived from the shell and is not produced by anaerobic or aerobic metabolism. While the clam is out of water a process resembling glycolysis in vertebrate tissue forms an acid. The acid is neutralized by the shell through the intervention of the living mantle. Part of the CO_2 dissolved from the $CaCO_3$ of the shell remains in solution. Gains in CO_2 and in non-volatile weak acid are equivalent to gain in calcium, so that these are the only participants in buffering. The whole process depicts the well-regulated use of calcareous shell for buffering the products of glycolysis during a period of anaerobic metabolism.

Tension output of muscles in hypotonic solutions. Alexander Sandow and Kenneth Moritz.

These experiments extend the research of the following on the activity of frog muscle in relation to varying tonicity: Cooke, *J. Physiol.*, **23**: 137, 1898; Meek, *Am. J. Physiol.*, **17**: 8, 1906; Chao, *Chin. J. Physiol.*, **10**: 547, 1936. The effect of absorption of water from hypotonic solutions on the output of *Rana pipiens'* gastrocnemii was studied by a method analogous to that used by Saslow and Webster (*J. Pharmac. and Expt. Therap.*, **53**: 142, 1935) in their study of caffeinized muscles. The two gastrocnemii were quickly removed, weighed, and immersed in Ringer's solution for an hour. Kymograph records were then obtained of the tension developed in response to 10 maximal break induction shocks (1 per second). Only those mates whose difference in output (tension per gram) during this initial period was less than 10 per cent were retained for further testing. One muscle of a symmetrical pair was then placed in a hypotonic solution made by diluting Ringer with distilled water; the mate used as a control was placed in an isotonic mixture containing the same fractional part of Ringer as the hypotonic solution, but the remainder made up of isotonic sucrose. After soaking about 3 hours, the muscles were weighed again and then stimulated with maximal shocks as before until complete fatigue. The records were measured for maximal response tension and total tension output. Most of the experiments involved tonicities 0.9 and 0.8 that of standard Ringer. Control experiments showed that muscles in isotonic solutions containing up to 20 per cent isotonic sucrose and the remainder Ringer, developed the same output as their symmetrical mates in pure Ringer.

The results demonstrate that the maximal response (in general agreement with Chao) first increased with increased water uptake and then decreased. The greatest maximal response occurred at a water absorption of about 10 per cent of the muscles' initial weight and amounted to 130 per cent of the maximal response of the control. The variation of the total output followed the same general curve as the above, but the greatest output, also at 10 per cent weight increase, was 145 per cent of the control. Further experiments are planned to elucidate the mechanism of these phenomena.

The mechanism for the control of the intake and the output of water by the vacuoles in the marine amoeba, Flabellula mira Schaeffer. Dwight L. Hopkins.

The marine amoeba *Flabellula mira* Schaeffer can live equally well in sea water diluted 20 times with distilled water or in sea water concentrated several times. Water is eliminated from the cell by expulsion of food vacuoles which on being expelled are largely filled with fluid. The intake of water which causes the swelling of the vacuoles in actively feeding amoebae is due to the production and isolation into the vacuoles of a by-product of metabolism. This substance is isolated into the vacuoles, giving them an osmotic value at the time of their origin many times that of the surrounding protoplasm and medium. They consequently absorb water according to the laws of osmosis. When the amoebae are not feeding due to unfavorable conditions, this substance is isolated into the vacuoles in the form of visible granules, *condensation granules*, in which case it exerts no osmotic effect and the vacuoles remain very small and stationary as to size.

This substance, when precipitated in the form of granules, shows properties generally ascribed to mitochondria, staining with Janus green B and by other techniques for staining mitochondria. The granules are soluble in ether, chloroform and alcohol, but not in acetone. They are destroyed by reducing agents and preserved by oxidizing agents.

In actively feeding amoebae the rate of water intake and elimination by the vacuoles is inversely proportional to the concentration of the outside medium and directly proportional to the volume of the cell, thus indicating that the osmotically active substance in the vacuoles is produced in a constant quantity in a unit of time by a unit volume of protoplasm. In non-feeding individuals the water intake and elimination by the vacuoles are zero or nearly zero and do not vary with the outside concentration.

On the kidney of the crayfish and on the uptake of chlorid from fresh water by this animal. N. S. R. Maloeuf.

A thorough study is presented on the anatomy of the kidney of the American crayfish (*Cambarus bartoni*) and on the arterial supply to that organ. This study is made as an anatomical prelude to physiological investigations similar (it is hoped) to the celebrated ones made by Prof. Alfred N. Richards and his school on the amphibian kidney. The nephridial canal (which is about 3 cm. long and a little over 1.5 mm. at its greatest diameter) occupies by far the largest mass of the kidney. It is here shown for the first time that the cells of its proximal (ventral) half are markedly different from those of its distal (dorsal) half. Hans Peters (1935) was the first to note that the nephridial canal is composed of two types of epithelium but made no statement as to which parts of the nephridial canal these types correspond. The cells of the proximal half form flat pavement epithelium and exhibit no apparent secretory activity while those of the distal half are large, columnar, and display large clear secretory vacuoles at their apices and a marked mitochondrial structure. This fact and the fact that the crayfish passively takes in fresh water through its gills (Maloeuf, 1937) makes it probable that the cells of the distal half absorb water from the blood and reject such into the lumen of the nephridial canal. This alone might account for the markedly hypotonic urine, i.e. a reabsorption of salts by the nephridial canal does not seem necessary. This appears especially probable since these animals are capable of absorbing Cl from fresh water by way of their skin (presumably by way of their gills). The epithelium throughout the kidney is monolayered.

When in fresh water, crayfish do not take in water by way of their mouths. The animals (*C. bartoni*) shipped from Wisconsin to Woods Hole in dry boxes (and which had thus remained about a week without water) were placed in running aerated tap water without food for about 10 days. At the lapse of this time and following they were found to exhibit a net excess of Cl absorption from tap water (a microburette run by a reversible telecron motor was used); i.e., they were absorbing more Cl than they were losing by way of their kidneys. The net excess was at first about 0.35 mg./20 gm./24 hours. The net excess fell with time and, in 20 days after the first analysis, it was about 0.048 mg./20 gm./24 hours. When their fast (of at least a month) was broken by a meal of minced raw beef the rate of excess Cl absorption declined slightly but significantly. These facts indicate that the net excess of Cl absorption is a compensation for the loss of Cl which had presumably occurred by way of the kidneys when the animals were in the dry boxes and thus unable to make up for the loss by an absorption of Cl through the skin. This discovery, while merely due to matters of circumstance, appears to be capable of offering important clues as to whether filtration is of importance in the renal function of the crayfish. When kept out of water, how long will the crayfish kidney secrete urine at a constant rate and how does this rate vary with the hydrostatic pressure of the blood? Except for the bladder and the proximal half of the nephridial canal all portions of the crayfish kidney exhibit secretory activity and nowhere is there anything resembling the tenuous epithelium of Bowman's capsule of the vertebrate kidney. With the discovery of a marked carbonic anhydrase activity in the gills of invertebrates (Ferguson), Cl absorption may also be of importance in supplying the Cl shift for the escape of CO_2.

The osmo-regulative function of the alimentary tract of the earthworm, and on the uptake of chlorid from fresh water by this animal. N. S. R. Maloeuf.

When an earthworm (*Lumbricus terrestris*) has been in tap water for some time (about 12 hours) and is weighed at successive relatively short periods thereafter it invariably loses weight until a certain minimum (about 15 per cent of the initial) is attained. The initial weight is completely regained or even exceeded if the worm is left undisturbed for several hours. These observations of Adolph (1927) have been confirmed. He suggested that the water is absorbed through the skin, is collected in the gut, and is expelled by way of the anus. By ligating the anterior and posterior ends of worms which had been in tap water for a protracted period and of worms directly before their introduction into tap water and by studying the weight changes it is possible to show that water which passes into the gut is expelled by both the mouth and the anus with approximately equal ease. As shown by the swollen condition at the anterior end and by punctures into the gut, the excess water is evidently partly stored in the crop. Further proof that water can be expelled from the mouth is shown by the sudden decrease in the net weight of the worms upon removal of the anterior ligature. A jet of clear colorless liquid was not infrequently noted to issue from the mouths of worms which had been in tap water upon ligating the anterior end. The percentage weight increase is greatest in worms which were ligated at both ends directly before their introduction into tap water from the soil. The weight then generally attains an approximately constant value (with minor fluctuations which can be due only to loss of liquid by way of the kidneys, the loss of which could be clearly observed under a binocular and which occurred from no other surface of the body) of approximately 115 per cent of the initial weight indicating that water is no longer absorbed. Dying worms showed no such constancy but continued to increase in weight. Amputating the nerve cord in the anterior end acts in the same manner as an anterior ligature.

The percentage of water in the gut contents of worms which had been in tap water for several hours is markedly greater than that in the soil from which the worms were removed. The percentage is still greater in worms in tap water and ligated at the posterior end; and is greatest in worms ligated at both ends. Correlated with this, the total quantity of materials (mainly water) in the gut of worms in tap water was much greater in worms ligated at both ends than in worms which were not ligated and still greater than in worms in the soil.

Worms, ligated at both ends to eliminate possible interference from the gut, will decrease in weight in even hypotonic sodium chlorid, urea, or glucose (added to tap water). The decrease is not necessarily proportional to the concentration of any one of these compounds. It is clear that no simple mechanism is involved. Per equimolar concentration, the decrease in urea is markedly less, this presumably being due to the greater penetrability of urea. With similar treatment the worms increase in weight in tap water.

Earthworms which had been in tap water for about 12 hours and which were then ligated at both ends showed a net excess absorption of chlorid from tap water. When ligated at both ends directly before introduction into tap water from the soil, all the worms showed, on the contrary, a loss of chlorid. This, together with the facts that worms so treated were especially viable and that untreated, apparently normal worms frequently died within 12 hours after introduction into running aerated tap water while all controls in moderately damp soil remained vigorous, indicates that the transition from soil to fresh water is not an easy one for the earthworm.

Earthworms introduced into tap water without any ligatures show but a slight net excess absorption of chlorid. This together with the fact that the unhealthy ones lost chlorid is nice evidence for the active retention of chlorid by the skin.

The kidneys probably play only a minor rôle in the osmo-regulation of the earthworm.

Development of half-eggs of Chaetopterus obtained by centrifugal force. Ethel Browne Harvey.

The *Chaetopterus* egg can be broken into two spheres of constant size by centrifugal force if suspended in a sugar solution. The white sphere contains oil, clear layer, nucleus or polar spindle, mitochondria (granules staining purple with methyl green) and a few yolk granules; the yellow half contains only yolk granules. The measurements are

	Diameter μ	Volume μ^3
Whole egg	94	434,900
White half	83	299,400
Yellow half	64	137,300

Both the white and yellow halves can be fertilized, and also can be activated artificially with KCl. The white half, both fertilized and parthenogenetic, forms polar bodies and cleaves according to the peculiar and typical annelid pattern. At first cleavage, it divides into two unequal cells, and at second cleavage, the smaller cell divides equally and the large cell unequally into a large and an intermediate-sized cell. It tends to become, especially the parthenogenetic white half, amoeboid, and often has large nuclei and irregular cells. Both the fertilized and parthenogenetic white half develop into swimming blastulae.

The yellow half, both fertilized (fertilized merogone) and parthenogenetic (parthenogenetic merogone), usually cleaves only once into two equal or unequal cells. It becomes amoeboid and develops no further.

The question of whether the delay in cleavage of Arbacia eggs produced with X-rays is caused by a general slowing of the cleavage process or by a block at some particular stage. Paul S. Henshaw.

Arbacia punctulata sperm have been exposed to doses of x-rays sufficiently large to cause a marked delay in the onset of the first cleavage (when allowed to fertilize normal eggs). Samples of eggs so fertilized together with controls (i.e., normal eggs fertilized with normal sperm) were fixed, sectioned and examined to determine at what stage during the cleavage cycle the slowing occurred. Similar studies were made in which the radiation was applied to the eggs rather than to the sperm.

Examination of a large number of organisms from a wide variety of samples disclosed the following: that about the same effects were obtained irrespective of whether sperm or eggs were treated; (2) that the entrance of the sperm and the fusion of the pronuclei take place quite as rapidly in case of the irradiated material as in case of the non-irradiated; (3) that the entire remainder of the cycle is slowed, but considerably more during the phase between the fusion of the pronuclei and the appearance of chromosomes; and (4) that if this phase of greatest delay be divided into two parts—the first from fusion of the pronuclei to the appearance of asters which attend mitosis and the second from the appearance of these asters to the appearance of chromosomes—definitely more delay is produced in the second part. The period of greatest delay is therefore associated with the prophase of mitosis and the manifestation of the greatest irradiation effect so far as delay in cleavage is concerned occurs when the nuclear material is undergoing changes in preparation for the formation of chromosomes.

The resistance of fertilized Arbacia eggs to immersion in KCl and NaCl solutions. E. L. Chambers and Robert Chambers.

The experiments performed consisted in immersing fertilized *Arbacia* eggs in isosmotic solutions of NaCl and KCl. The procedure differs from those of previous investigators in that the eggs were passed through many washes of the given salt to eliminate all traces of sea water and were left continuously in the salt solution. Temperature and pH were carefully controlled.

Washed eggs were immersed in the solutions at varying intervals after insemination. The effect was noted by the percentage of cleaving eggs. The toxicity of the NaCl-solution was very great and was directly proportional to the length of sojourn in the solution. Division occurred only among those eggs immersed shortly before or during the first cleavage period. Eggs immersed in KCl-solution exhibited a reverse effect. Those immersed shortly after insemination were found to be highly resistant and the percentage of cleavage fell rapidly to zero when immersed at any time later than 10 to 20 minutes after insemination.

The resistant period to K-ions has several characteristics: (1) it varies greatly in batches of eggs from different sea-urchins, (2) it occurs only at one stage in the cycle of development of the fertilized egg, there being no resistant period between the first and second cleavage, (3) it is not related to the cycle of mitosis, nor to the time of union of the pronuclei and (4) its length is independent of pH, temperature (15–25° C.), and the aging of the eggs.

Unfertilized and fertilized eggs are highly and equally susceptible to Na-ions. To K-ions, the unfertilized and fertilized eggs are highly resistant from 5 to 12 minutes after insemination. This indicates that in so far as K-ions in the medium are concerned the cytoplasm does not change from the unfertilized to the fertilized state until many minutes after insemination.

Indophenoloxidase in Arbacia eggs and the Nadi reaction. Albert E. Navez.

In a previous note by A. E. Navez and Ethel B. Harvey published here in 1935, measurable differences of indophenoloxidase activity of the unfertilized eggs of *Arbacia* intact, stretched and halved by centrifugalization were reported, which for equivalent volumes of material was: whole egg (1.0); white nucleated half (2.4); whole egg stretched (3.2) and red enucleated half (4.8) in arbitrary units.

In an attempt to determine the basis for the increase in activity of the stretched eggs, experimental evidence brought some doubt on the reliability of the Nadi reagent as a specific indicator for the indophenoloxidase, especially in view of the common use of indophenol blue by plant cytologists, as a stain for oleoplastic inclusions.

In an effort to detect some substance(s) reacting in the same way, whole eggs were cytolized, put immediately in contact with fat solvents (chloroform, benzene, ether, etc.), the solvent separated on the centrifuge (in closed tubes) and distilled off in vacuum at room temperature. An oily residue of high viscosity, dark red color (probably by denaturation products of echinochrome) and easily oxidized was left behind. Preliminary experiments showed also an increase in the rate of oxidation on addition of iron salts.

This "oil" (crude extract) is saponifiable (with index of saponification around 200) and fixes iodine easily (index of iodine 180–190), indicating the presence of double bonds and giving a basis for the notion of autoxidation. Both of these values are preliminary and may be corrected later on further purification of the "oil."

The most important characteristic is its reactivity with the Nadi reagent, even after warming it in vacuum for several minutes, in a boiling water bath. This, coupled with its extractability, makes the present reaction exclude the specificity of the Nadi reagent for the oxidase.

It may be inferred that this oil is actually bound to a protein when one takes into account the observation of Heilbrunn on the action of ammonium salts on the size of the oil cap in centrifuged eggs; the possibility that the unstable solution of paraphenylenediamine may contain, by secondary reactions, traces of ammonia and that on stretching eggs evidence has been found that some ammonia (or a compound able to give off ammonia) may be liberated.

The presence of an "oil" easily oxidizable brings in a new factor in the scheme suggested by Korr for the respiratory system of *Arbacia* eggs.

An oxidase determination can be made by correcting for this "oil": determinations are done successively on living cells and on killed cells (by heat).

Figures were reported respectively for the "oil" and "oxidase" parts of whole, stretched and halved cells. Evidence points towards an increase in oxidase activity in stretching of cells as well as to an increase of the reactant "oil."

Electric impedance of nerve during activity. K. C. Cole and Howard J. Curtis.

Alternating current impedance measurements have been made over a wide frequency range on the giant nerve fiber from the stellar nerve of the Woods Hole squid, *Lolego pealii.*[1] Narrow electrodes were placed on either side of the fiber, so that the current flow was perpendicular to the fiber axis. In order to observe and measure the change in transverse impedance during the passage of the nerve impulse, the amplified output of the Wheatstone bridge was applied to a cathode ray oscillograph, and as a sweep circuit moved the spot across the screen, there was a narrow horizontal trace when the bridge was balanced. When the nerve was stimulated at one end, the line broadened to a band as the impulse reached the impedance electrodes, indicating a bridge unbalance, and returned during the recovery phase.[2] By an appropriate analysis, it was found that during activity the membrane phase angle is unchanged, the membrane capacity decreases very slightly, while the membrane resistance drops from probably about 5000 ohm cm.^{2} or more to an average of 25 ohm cm.^{2}

The onset of the resistance change occurs somewhat after the start of the action potential, but coincides quite closely with the point of inflection on the rising phase. At this point the membrane current density reverses in direction, corresponding to a decrease of membrane electromotive force, so that this e.m.f. and the conductance are closely associated properties of the membrane, and their sudden changes are themselves, or are due to, the activity which is responsible for the propagation of the nerve impulse.

These results are qualitatively identical to those found for the single plant cell *Nitella* during activity.[3] Furthermore, the resting impedance properties of nerve fibers from several different animals have been measured [4] and found to be very similar to those of the squid fiber, so it is reasonable to suppose that similar changes occur in all nerves during excitation and conduction.

The vibratory response of the skin and its relation to pressure sensitivity. Frank A. Geldard.

Considerable confusion exists at present as to the essential nature of vibratory sensations. A widespread view is that they are mediated by a separate "vibratory sense," having its own receptors and nervous pathways and hence constituting a sense modality *sui generis.* Contrasting is the theory that vibratory senastions are but perceptual patterns of superficial touch and, as such, are but "touch in movement." Within clinical neurology the vibratory sensation is conventionally regarded to be identified with "deep" or periosteal sensitivity and the tuning fork test is an important part of the clinician's routine. The strongest evidence from clinical neurology, thus far quite uncritically evaluated, is the apparent dissociation of touch

[1] Curtis, H. J. and Cole, K. S., *J. Gen. Physiol.*, **21**: 757, 1938.

[2] Cole, K. S. and Curtis, H. J., *Nature*, **142**: 209, 1938.

[3] Cole, K. S. and Curtis, H. J., *J. Gen. Physiol.*, Sept. 20, 1938.

[4] Cole, K. S. and Curtis, H. J., Cold Spring Harbor Symposium on Quantitative Biology, **4**: 73, 1936.

and vibratory sensations in tabes, multiple sclerosis, syringomyelia, and other nervous affections.

Qualitative experiments by von Frey (1915), identifying vibratory reception with the operation of the "pressure sense," never adequately harmonized with other findings, point the way to critical quantitative determinations of the relations between vibration and pressure. On the volar surface of the arm there were located two populations of touch spots, one highly sensitive to pressure stimulation, the other quite insensitive. Controlling mechanical skin gradients, the frequency, amplitude, and wave-form of the movements of an oscillator-driven needle (Geldard and Gilmer, *J. Gen. Psychol.*, 1934), and observing skin movements by slight off-tuning of intermittent illumination in the microscope field, the vibratory thresholds of all spots were measured for the frequencies 64, 128, 256, 512, and 1024 cycles. Pressure-sensitive spots prove to have uniformly low vibratory thresholds (of the order of 25μ amplitude), while insensitive spots have high (100–250μ) thresholds and show great variability. The essential identification of vibratory and pressure sensitivities is held to be demonstrated. Further, the consequences of the widespread conduction of forced vibrations by the skin and other tissues for the interpretation of various clinical anomalies are indicated.

Reversal of phototropic reaction in Daphnia by the use of photosensitizing dyes. E. Alfred Wolf.

The property of vital stains to act as photosensitizers in light was applied to the study of phototropic reactions. Positively phototropic specimens of *Daphnia longispina* were stained in solutions of rose Bengal and eosin for three to twelve hours. Their movements in white and filtered light were observed in a glass trough covered by a black cardboard hood that left one side of the trough open to light; a slit in the top of the hood allowed observation of the animals. The animals were introduced in the middle of the trough in groups from six to nine, or singly either at positive or negative end, and their position was recorded after definite periods of time.

In a first series red and green filters of glass were used, the red absorbing those wave-lengths that would be absorbed by the stained animal itself and consequently injure the animal; the green letting those injurious rays pass that now can act on the animal. Animals were tested in groups. In red the majority accumulated in the positive half of the trough within five minutes of exposure; change to green or white caused the animals to move to the negative half, with occasional tardy specimens still in the positive half after five minutes.

In another series the path of single specimens was recorded in one-minute intervals. Liquid filters of solutions of the dyes themselves were used thus assuring complete absorption of the harmful rays; animals remained in the positive half of the trough or migrated to it if released at the negative end. After thirty minutes the filter was removed and the animals very quickly fled to the negative end. After six to twelve minutes the animals started to return to the positive end and died one to three minutes after arrival.

Motion picture showing microscopic changes in fibers of cardiac and skeletal muscle of invertebrates and vertebrates during contraction, retraction, and clotting. Carl C. Speidel. (See also abstract of paper on p. 349.)

These cinéphotomicrographs reveal many of the histological changes that accompany contraction, retraction and clotting in fibers of striated muscle. Some of the pictures were taken directly from the intact living animal; others were taken from excised tissues suitably mounted for microscopic examination. Among the activities recorded are the following: in *Palaemonetes*—contraction and folding of fibers during electrical stimulation, the resting state followed by steady and sustained contraction of one portion of a group of fibers, and uncoördinated localized contraction nodes traveling along several fibers; in *Limulus* leg muscle—traveling contraction nodes somewhat coördinated on three adjacent fibers, collision of nodes, nodes in fibers with retraction clots; in *Limulus* heart musle—fiber showing rhythmic contraction node formation at same locus, node origin followed by travel toward one end, retraction clot barrier to contraction node advance, node formation induced by electrical stimulation, barrier of sharp bend in fiber to node advance, rhythmic node formation in heart fibers with retraction clots one day after severe heart injury, contraction node advance to a slight barrier and its division, followed by the reflection backward of one part and the advance of the other, elimination of barrier by electrical stimulation; cardiac fibers in the intact living *Polyonyx*, also in the heart of *Loligo* and *Venus*, beating lymph heart of frog tadpole, retraction clots and monocyte movements in fibers of starved tadpole, and retracting fibers of striated muscle in the swim-bladder of the sea robin.

Some of the pictures also portray the various responses to injury of fibers of cardiac and skeletal muscle of frogs, frog tadpoles, and rats, a detailed description of which has recently been published (*Anat. Rec.*, **70**, Suppl. No. 3, p. 102).

The action of direct currents on the cell nucleus (Film). W. R. Duryee.

This film has been prepared to show the movements ot chromosomes and nucleoli within an interkinetic nucleus as well as to study the colloidal structure. Only in this way can the kinetic aspects and three-dimensional relationships be comprehended.

Nuclei, both isolated in Ca-free Ringer and *in situ* in transparent young cells, are from the ovarian eggs of the salamander *Triturus pyrrhogaster*. The material is mounted in 0.05 cc. drops through which 10–15 milliamperes of current from a 45-volt battery is passed.

Chromosomes and nucleoli always move toward the anode (+), reversing with the current twenty or more times, thus showing that they are negatively charged. While in the center of the nucleus they appear to move as a unit inasmuch as they are imbedded in a weak elastic gel, the Binnenkörper, which contributes to the formation of the maturation spindle. Proof ot this elastic matrix is given by the fact that chromosomes and nucleoli spring back as soon as the current stops flowing, after they have been pressed against the inside of the nuclear membrane.

Similar effects are shown in intact transparent eggs. In addition, the nucleus as a whole moves toward the anode, thus indicating its negative charge, while cytoplasmic granules move toward the cathode. Darkening and contraction of the nuclear membrane toward the anode is interpreted as adsorption of cations from the cytoplasm on this side, while it may be seen that the opposite side, toward the cathode, swells and eventually dissolves due to the accumulation of anions. The latter effect is reinforced by the wave of hydroxyl ions spreading from the cathode.

Hydration and dehydration of follicle cell nuclei (Film). W. R. Duryee.

In this film kinetic relationships between environment, cytoplasm, and nucleus are demonstrated. Tangential views of the double layer of follicle cells on young *Triturus* ovocytes give both sequential data on swelling and shrinking and also cytological evidence on the structure of somatic nuclei.

Gradual dilution of the isotonic Ringer by distilled water makes the nuclei swell and become hyaline. On the other hand, shrinkage by hypertonic Ringer causes chromosome-like bodies to appear. These 20–30 presumable chromosomes are oriented vertically to the egg surface and condense more heavily on the side toward the egg cytoplasm. Since this process is freely reversible it furnishes inferential evidence on the interkinetic persistence of the chromosomes.

AUGUST 31

The function of muscles in locomotion. Herbert Elftman.

The function of the leg muscles in walking can be studied by recording the force which the foot exerts against the ground by means of an especially designed apparatus, a motion picture of the individual being taken simultaneously. From these data the torques which the muscles exert on the trunk, shank, thigh and foot can be determined and the rate at which they do work can be found. Tension is necessary in the muscles, even when the muscle tissue does no physical work, for two purposes: (1) putting other forces into equilibrium so that the desired movement is obtained; (2) transmitting energy from one part to another by "tendon" action. Muscles regulate the energy level of the locomotor mechanism by receiving energy and by liberating energy. In walking this occurs in two cycles in the muscles of each leg for each double step. This alternate reception and release of energy by the muscles suggests the possibility of energy storage. If no storage occurs, and no account is taken of other energy requirements, the efficiency of the leg muscles in dissipating energy and mobilizing it from chemical sources would be at least 39 per cent, using the figures of Atzler and Herbst for oxygen consumption during walking. The partial storage of energy by the muscles is indicated. The possibility of effective storage depends not only on the characteristics of muscle tissue, but also on the disposition of the muscles. All of the functions of the leg muscles in walking could be served by a single muscle passing over three joints. The actual muscles present represent a compromise between such a muscle, with its efficiency, and a system of one-joint muscles with greater adaptability to different types of movement.

The effects of copper and of vanadium on the frequency of division. William J. Bowen.

Chilomonas paramecium can be grown indefinitely in a solution containing only simple compounds and under environmental conditions easily and accurately controlled (Mast and Pace, 1937, *Jour. Cell. and Comp. Phys.*). It therefore offers an excellent opportunity to study the effects of the heavy metals on the growth of a protozoan.

The effects of copper, in $CuCl_2$, and of pentavalent and tetravalent vanadium, in Na_3VO_4 and $VOCl_2$ respectively, were investigated by ascertaining the rates of division during 15 consecutive days of four isolated lines of *Chilomonas* grown in culture fluid to which various amounts of the above compounds were added.

The average number of divisions of the four lines indicates that $CuCl_2$ in any concentration between 3×10^{-7} and 1×10^{-20} molar has no beneficial effect on the rate of growth of *Chilomonas*, that in a concentration of 1×10^{-10} molar it retards growth, and in a concentration of 3×10^{-7} molar it kills the organisms.

No acceleration was obtained in culture fluid containing Na_3VO_4 in concentrations ranging from 1×10^{-3} to 1×10^{-12} molar, but the organisms grew in the relatively high concentration of 1×10^{-3} molar. However, in culture fluid containing tetravalent vanadium there was definite acceleration. As the concentration of $VOCl_2$ increased, the frequency of divisions increased from 3.54 per day in zero concentration to 3.91 in 1×10^{-10} molar and then decreased.

These results with the two forms of vanadium indicate that partially oxidized vanadium is beneficial for the growth of *Chilomonas paramecium* and that the completely oxidized form is not beneficial.

Sex balance in the progeny of triploid Drosophila. Sarah Bedichek.

Males from eight X–IV translocation stocks were crossed to triploids with recessive markers, producing sterile intersexual "duplication intersexes" with a short fragment of the X plus two complete X's and three autosomes and weakly fertile hypotriploid females with longer X fragments plus 2X3A. Duplication intersexes bearing interior regions of the X in triplicate were studied by combining right and left-hand fragments from two different translocations. Of eight duplication intersexes with very short sections, covering successively the entire X chromosome, in triplicate, none showed a marked shift in the female direction. Therefore a single primary sex gene can not exist capable of producing a functional female when represented three times with 3A regardless of the dosage of the remainder of the X chromosome. There was a graded shift toward femaleness in the phenotypic appearance of individuals with an X fragment plus 2X3A with increasingly longer fragments. Individuals were hypotriploid with either right or left-hand section of two translocations (t–lz and lz–v breaks). Furthermore, fertility of certain fragments plus 2X3A aneuploids begins before the last trace of intersexuality vanishes, since some hypotriploids with the right-hand fragment of the t–lz and lz–v breaks possess very rudimentary sex combs. For this reason and the fact that shorter sections from the left than from the right-hand region of the X chromosome plus 2X3A result in weakly functional females, we may conclude that the portion of the X to the left of the t–lz break seems a little more female potent in relation to its cytological length than the portion to the right of this break. A drop in temperature to 18° C. produces intersexuality in those individuals carrying the right-hand fragment of the t–lz break but does not alter the female appearance and function of hypotriploids bearing longer fragments.

The antihormone problems in the salamander. Eduard Uhlenhuth, James U. Thompson and Joseph E. Schenthal.[1]

In 1934 Anderson and Collip reported that after a few injections of Thyreo-activator (T.A.), the thyroid becomes refractive to this hormone, owing to the action of antihormones. Sor far we have been able to examine salamander thyroids removed after a maximum of 40 daily injections of T.A. extracted from beef A.L., but failed to observe refractoriness.

The activity of the thyroid was expressed by its colloid content calculated as "Colloid Level" (C.L.). Ringer extracts of A.L. were administered daily to adult animals of the California newt. In one experiment from 1 to 40 injections of 10 mg. of A.L., in another experiment from 2 to 33 daily injections of 50 mg. of A.L., in a third one 10, 34, 36, 38 and 40 injections of 50 mg. of A.L. were administered. In every case variations of the colloid content due to the cyclic alternation of colloid release and colloid storage are observed; but the values obtained in the beginning are not higher than those obtained at the end of the experiment. Thyroids taken after 26, 34, 36 and 38 injections have C.L. values of only 3 to 9 per cent, which are among the lowest values obtainable.

The O_2-consumption of animals of the same species, injected daily with T.A., was tested. It shows a rise while the colloid is released and a drop while it is stored, exhibiting the same wavelike variations as the colloid content but in opposite directions.

During a period of prolonged T.A. administration the thyroid is in a state of increased activity all the time, but at some time it releases colloid and at other time it manufactures and stores new colloid. No decrease in the response to T.A. was observed in the salamander up to 40 injections of T.A.

The effect of the sex-stimulating factor of the anterior pituitary gland on the testis of the bullfrog. Roberts Rugh.

Most of the maturation figures may be found in the seminiferous tubules of the bullfrog testis at any time of the year. Spermatozoa first appear at the time of metamorphosis but males are not considered sexually functional until they measure about 100 mm. in body length. Since spermatozoa are present, even though there is little or no sex-stimulating hormone present in the males of body length less than 90–100 mm., it is assumed that the pituitary *growth* hormone controls maturation.

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[1] From the Department of Anatomy, University of Maryland Medical School.

The anterior pituitary gland from mature males or females will effect marked changes in the testis of any bullfrogs, regardless of size or maturity. The procedure has been to excise and fix the left testis as the control and then to inject the frog with an adequate dose of pituitary tissue and to fix the right testis within 24–48 hours.

Since the anterior pituitary gland hormone seems to empty the testis of any loosely arranged cell products, such as the spermatozoa of the mature bullfrog and any (except the earliest) maturation figures of the immature males, it is suggested that it acts through the medium of the smooth muscle tissue so abundant in the testis. Primary spermatogonia, whether in the metamorphosing bullfrog or in the adult, are unaffected. This is probably due to the fact that they are an integral part of the marginal epithelium of the seminiferous tubule. Even the cortical pro-gonad of the prematamorphosing testis shows the effect of this hormone. It has previously been demonstrated that the anterior pituitary gland hormone not only causes the rupture of the ovarian follicle but stimulates the smooth muscle cells of the follicle wall to force the egg out through the rupture area. Further evidence of the activation of this smooth muscle tissue is the fact that if an adult testis is studied within 16–20 hours after the host has been treated with the hormone, only the peripheral seminiferous tubules will have been emptied. Final confirmation of this thesis will require the severing of the sympathetic connections with the gonad prior to anterior pituitary hormone treatment.

Studies on barnacle larvae. J. Paul Visscher.

Alcippe lampas is a boring barnacle which inhabits mollusk shells which are occupied by hermit crabs. It has been found in 45 per cent of the shells examined and one shell had 37 specimens in it. The animal is found completely embedded in a cavity in the shell. This cavity has a relatively small orifice through which the appendages of the barnacle are thrust for feeding and for respiration. The barnacle may attain a diameter up to one-half inch. It is disk-shaped and is usually 3 or 4 times greater in width than it is in thickness.

The larvae of this barnacle were found abundantly in June and but rarely in late July and August.

The eggs of this animal are large and full of yolk and the nauplii were observed to moult regularly without feeding. There are six nauplear stages—each persisting for about 24 hours under the conditions of the experiment. The cyprid larva is long, slender and very active. The activity is noticeably increased by the addition of a fluid extract of the tissues of the hermit crab.

Conclusions:

1. *Alcippe lampas* is a common barnacle in the Woods Hole region.
2. The nauplii pass through six stages or moults in about one week.
3. The nauplii do not feed during this period.
4. The cyprid larvae are activated by the presence of the hermit crab, *Pagurus policarus.*

The spawning reaction of Nereis limbata with emphasis upon chemical stimulation. Grace Townsend.

Alternation of light and dark can induce a corresponding alternation of swimming and quiescence suggesting that occurring in nature. With sufficiently strong light to induce rapid quiescence, this can be repeated at half-hour intervals. Tactile stimulation is necessary for achieving the quiescent state. Without a bottom layer of sand or provision for tactile stimuli, continuous swimming occurs in any illumination.

A vibration sense localized on the parapodial cirri is sensitized during sexual metamorphosis and enables the swimming worms to avoid collision. This sense is supplementary to the chemical sense in inducing circling orientation and under certain conditions may induce circling independent of chemical stimulation.

The response of the male is a typical sensory response and normally occurs only during stimulation. The female response possesses neuro-hormonal characteristics and may continue in the absence of stimulation. The male response may sometimes be artificially induced to resemble the female response.

A special sensitivity to egg-water, cystine, cysteine, and glutathione, not existing in the non-sexual phase, is developed on the epitoke of the male at sexual maturity. The sensitivity appears to be of great chemical specificity, extending even to the distinction of dextro and laevo forms of cystine.

A physiological completion of sexual metamorphosis occurs during swimming. The quantity of male-stimulating material is increased in the female and the sensitivity of both males and females to material of the opposite sex is increased. The processes involved are reversible.

Physiological assays concerning the nature of fertilizin. Grace Townsend.

The male of *Nereis limbata* has been hypothesized by Lillie and Just (1913) to spawn in response to material from the egg which is neutralized by the sperm and is essential to fertilization. *N. limbata* males are sensitive to 1 : 1,000,000 parts glutathione. Contrastingly they do not spawn in response to unrelated sulphydryl compounds, various amino acids, iodides, hormones, enzymes, histamine, acetyl choline, and miscellaneous compounds tested. Marine eggs contain from 0.03 to 0.04 per cent glutathione wet weight. A reducing substance as tested by the very sensitive ortho-chloro-phenyl-indophenol leaches from the eggs.

The material of egg-water and glutathione possesses all investigated properties in common: location in the egg, solubility in water, precipitation by acetone, stability in acid, lability in alkali, dialyzability, destruction by aging, boiling, irradiation, copper, arsenic, and monoiodacetic acid, lack of destruction by NaCN, adsorption by kaolin and charcoal, apparent inactivation by sperm, blood, and cytolyzed egg, increased physiological potency in the induction of spawning in a slightly acid medium. These properties, in so far as covered by the studies of Woodward (1918) and Glazer (1921), are in agreement with those ascribed to the parthenogenetic fraction of "fertilizin." They differ from the properties assigned to the agglutinin fraction and from the properties assigned to "fertilizin" by Lillie (1913) with respect to dialyzability and degree of lability in the presence of heat and upon aging. For a given molecular concentration, the material of egg-water is more potent than glutathione.

The fertilizability of washed eggs is partially restored by immersing the eggs in 1 : 3–5,000 parts glutathione.

A study of the integument of the squid, during staining with methylene blue. Elbert C. Cole.

The selectivity of methylene blue for various tissue elements was found to be greatly increased by the addition of appropriate substances to the staining solution. A living squid was placed in a screw-cap jar and the jar nearly or quite filled with a standard solution of one part of certified methylene blue in 20,000 parts of sea water. The jar was sealed and staining under anaerobic conditions allowed to proceed for an hour or more. Upon exposure of the squid to air, nervous elements of the integument were found to be stained. The addition of 1 per cent of methenamine (the brand "Urotropin" was used) to the standard solution increased selectivity for nervous tissue. The addition of KCN, one part in 200,000, together with ethyl urethane, one part in 300, to the standard solution produced highly selective staining of the radial muscle of the chromatophores.

With these solutions a study of the structure and activities of the various components of the integument was made. The action of the radial muscles during movement of the chromatophores, the innervation of the chromatophores, the attachment of radial muscles to the connective tissue framework of the skin, and the stresses and strains produced on this framework by violent contractions of radial muscles were studied. It is suggested that these methods may be generally applicable to the study of structure and function of particular cells in complex tissue masses.

The use of the clam heart as a test object for acetylcholine. Carl C. Smith and Louis Levin.

A method is described for the use of the heart of the marine clam, *Venus mercenaria*, as a test object for acetylcholine. Details of the procedure for isolating the heart are given and a special chamber adapted to the purpose is described.

The sensitivity of the heart to choline was studied and the smallest concentration found to give a demonstrable inhibition was 1 : 50,000. Acetylcholine in a concentration of 1 : 1,000,000,000 produced a 50 per cent reduction in amplitude of the beat from which the heart recovered quickly. In solutions containing choline and acetylcholine, in which the choline is present in a subminimal quantity, no effect upon the normal inhibition produced by acetylcholine was observed. Sensitization by eserine is not needed. With this preparation in the apparatus described, the least amount of choline which can be detected is 0.2 mg.; the smallest amount of acetylcholine which can be assayed is 0.00001 mg. or 0.01 gamma.

The fact that the clams are easily available and can be kept for long periods makes the use of this preparation advantageous as a simple tool for the bioassay of choline derivatives.

The elimination of molds.[1] Oscar W. Richards and Katherine J. Hawley.

Eighteen different species of mold grow profusely in the Chemical Room of the Marine Biological Laboratory during the summer when the relative humidity exceeds 80 per cent. The molds grow on almost everything except glass and metal and during the last five years experiments have been made to eliminate the mold growth in order to prevent damage to equipment and materials. Twenty-one chemicals were tested and most of them were ineffective under the severe condition encountered. The most promising fungicides are thymol, cinnamon oil, α-nitronaphthalene and Phenox. Seasonal protection of most materials is possible with these chemicals and concentrations are recommended for their use. Longer protection may come by combining these or more potent chemicals with a suitable binding varnish which will hold an effective concentration of the fungicide on the surface of the material to be protected.

A comparative study of water aspirators. S. E. Pond, E. P. Little, A. M. Smith and J. D. Graham.

Devices in use for simple evacuation and for air lifting, by means of water pressure, vary considerably: (*a*) final vacuum, (*b*) air volume moved, or speed of evacuation, and (*c*) water consumed in operation. Some units have been found to take larger quantities of water for effective operation than appears necessary and are therefore unusually costly for general use. In tests reported, a two-liter flask was evacuated, with water operated at 40 lbs. per square inch, permitting a group classification of the aspirators which were available:

I. Vacuum established and maintained in 60 seconds.

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[1] The detailed report of this work has been accepted for publication in the *Jour. of Chem. Educ.*

II. Maximum vacuum reached in time indicated, with water consumption (expressed as cubic feet per minute).

III. Effectiveness with an arbitrary but uniform aperture (air intake) during operation, indicated by column of mercury in cms. lifted during operation, at equilibrium.

Twelve different aspirators were studied representing seven different makes:

Group No.	Vacuum 60 sec.	Water cu. ft./min.	Final Vac.		Lift cm.	Composition
			Cm.	Time		
A 1	74.2	0.69	74.2	55″	33.0	Stainless steel
2	72.0	0.54	74.2	2′	15.0	Brass
3	71.5	0.45	73.8	2′	20.0	Brass
B 4	64.5	0.21	74.3	5′	9.3	Stainless steel
5	61.5	0.27	74.2	5′	6.6	Brass
6	61.0	0.26	72.5	5′	10.3	Brass
C 7	59.5	0.57	69.3	5′	5.1	Stainless steel
D 8	57.1	0.24	72.7	7′	10.1	Glass
9	54.8	0.17	73.7	7′	4.7	Brass
10	50.5	0.26	72.2	7′	6.9	Glass
11	48.0	0.21	74.0	7′	4.3	Glass
12	48.2	0.21	72.9	5′	4.9	Brass

The first two groups differ appreciably in speed of operation and markedly in water consumption; but group "C" is of still slower speed with very high water consumption. Pump No. 1 is the largest obtainable, essentially for air pressure work where motor-driven pumps are not available and would be used where extreme speed is required for water power. The last group, "D," indicates the most economical for moderate speed under the conditions of the tests, and includes the glass pumps which appear most useful for handling corrosive vapors when insufficiently trapped.

All pumps have been operated with the check valves as supplied in spite of the fact that these corrode easily and have an effect upon speed of evacuation. Other points of design, related to effectiveness in operation, are under consideration.

Papers Read by Title

The effect of electric current on the physical consistency of sea urchin eggs.

C. A. Angerer.

This investigation is one of a series undertaken to show the effect of various stimulating agents on protoplasm. These experiments have been made primarily with the view of continuing the study on protoplasm of some phylogenetic different species from that used heretofore, namely, Amoeba. The physical consistency was determined by the centrifuge method. The material used was the unfertilized eggs of the sea urchin (*Arbacia punctulata*). The stimulating agent was electric current (direct and alternating). The eggs were subjected to electric current through a Zn/$ZnSO_4$/sea water-in-agar system which in turn was in circuit with either the A.C. or D.C. service line running into the laboratory. Relatively strong current densities had to be employed in order to affect noticeably the physical consistency of the eggs. A current density of 0.005 amperes/cm.^{2} cross-sectional area of available electrode surface was employed for both alternating and direct current. When plotting the relative "viscosity" as a function of varying intervals of time (in seconds) during which any given quantity of eggs are exposed to the current density in question, there is during the first 2 seconds of current flow a progressive decrease in "viscosity." With continued application of the current the "viscosity" levels at a minimum which is 66 per cent of the control value. With still further application of the current the "viscosity" begins to increase after 5 seconds of exposure at the linear rate of approximately 10 arbitrary units/second. The experiments were discontinued after 15 seconds. The experimentally induced changes in "viscosity" are reversible and on insemination of the eggs normal membrane elevation and cleavage occur. These results on electrical stimulation are in line with those already obtained for the action of this and other stimulating agents (mechanical agitation, heat and ultra violet radiations) on Amoeba and sea-urchin egg protoplasm.

The effect of sodium azide on the frequency of the embryonic Fundulus heart. C. W. J. Armstrong and K. C. Fisher.

Recently Keilin (*Proc. Roy. Soc.*, B, **121**: 165, 1936) has shown that many of the effects of cyanide in cell respiration are duplicated by sodium azide. Since it has been shown that the effect of cyanide on heart-beat frequency in embryonic fish can be quantitatively characterized, it is of interest to use the heart-beat frequency as a quantitative test of how far the similarity between cyanide and azide extends.

Eggs of *F. heteroclitus* (from 3 to 7 days after fertilization) were irrigated with aqueous solutions of sodium azide of concentrations ranging from .004M to .00002M. The solutions were approximately neutralized (phenol red) to a pH of about 7.2 by the addition of a few drops of .1N NaOH. Normal frequencies were obtained by observing 12 to 16 organisms in a tube in running distilled water. The distilled water was then replaced with azide solution and the consequent frequency decline observed until a new frequency level had been attained (= inhibition level). Occasionally this level was observed to persist for many hours. The maximal inhibition level (= 47 per cent normal frequency) was obtained with solutions of a concentration greater than .001M.

By plotting log [azide] against log $\frac{\text{per cent inhibited frequency}}{\text{per cent uninhibited frequency}}$ (where per cent inhibited + per cent uninhibited frequency + maximal inhibition level = 100 per cent of normal frequency) a linear relation between the two appears indicating that the data can be described by the Mass Law.

The degree of inhibition depends upon the pH. The following results were obtained using a .0001M sodium azide solution.

pH	Inhibition Level (as per cent normal)
8.0	100
7.3	80
6.0	50

Cf. the effect on yeast etc. (Keilin, 1936).

A comparison of the effect of azide on heart-beat frequency in *Fundulus* with that of cyanide (the latter from the work of K. C. Fisher and R. Öhnell) is given.

	Azide	Cyanide
1. Maximal inhibition level (M.I.L.)	47	27
2. Weakest solution to produce (M.I.L.)	.001M	.001M
3. Time to attain M.I.L. using 2	5–7 hrs.	4 hrs.
4. Slope (*n*) of line from log plot	1.3(5)	1.6

Reducing activity of fertilized and unfertilized Arbacia eggs. Robert Ballantine.

The ability of *Arbacia punctulata* eggs, fertilized and unfertilized, to reduce methylene blue (also cresyl blue and toluene blue) has been studied under comparable conditions in a nitrogen atmosphere and the results expressed as the mm. of the oxygen equivalent of methylene blue reduced per hour per mg. N_2 in the eggs. On the average, 1 cc. *Arbacia* eggs contains 28.5 mg. N_2. Fertilized eggs reduced methylene blue 233 per cent to 93 per cent more rapidly.

The reduction rate does not change from 10 minutes to $1\frac{1}{2}$ hours after fertilization and the sperm which are used to fertilize the eggs are not responsible for the increased reduction time: neither do fertilized or unfertilized eggs give off anything to the medium which has reducing power. Blocking iron systems with Na azide does not affect reducing time in either fertilized or unfertilized eggs.

Addition of substrate (e.g. lactate) to either fertilized or unfertilized eggs does not change reducing time, presumably because lactate does not penetrate. If cytolyzed in 50 per cent sea water without washing, reduction is much more rapid, especially in unfertilized eggs. If cytolyzed and washed, reduction is very slow but on adding lactate reduction increases more than control uncytolyzed eggs, whether fertilized or unfertilized.

Rate of absorption of both methylene blue and cresyl blue by unfertilized and fertilized eggs is the same under aerobic conditions. Methylene blue penetrates much less rapidly aerobically than it can be reduced in the medium under unaerobic conditions. The change to leuco dye within the cell must maintain a very steep diffusion gradient.

Study of per cent reduction (determined electrometrically)—time curves indicates that permeability to dye cannot entirely explain the greater reducing time of fertilized eggs but that their behavior may be connected with increase of available substrate or of active dehydrogenases.

Studies on the mechanism of growth in Planaria maculata. Ludwig von Bertalanffy.

In continuation of a quantitative theory of growth, as developed in former investigations of the author (cf., for instance, *Human Biology*, **10**: p. 181ff.), some basic factors involved in growth have been investigated in *Planaria maculata*. As suggested by Hesse and others, and quantitatively formulated in the author's theory, it may be assumed that growth is controlled by the fact that in the beginning the magnitude of resorbing surfaces, and, therefore, the quantity of building material per unit mass is relatively high; but that the latter becomes gradually smaller due to the fact that the relative size of interior surfaces decreases, leading thus to a decrease of growth rate and finally to an equilibrium in which the resorbed materials only maintain the balance, and growth stops. This hypothesis has been tested, for the first time, in a quantitative way. Measurements of the *magnitude of the surface of the intestinal tract* have been made by various methods. It was found that: (1) the length of the intestinal tube increases many times with increasing size of the body; (2) that the same holds for the surface, but that (3) the relations of length of the intestinal tract: weight, and interior surface : weight continually decrease; (4) the relation of the magnitude of interior surface: weight $\frac{2}{3}$ remains approximately constant over three orders of magnitude, some hundredths of a milligram in newly-hatched individuals to some twenty mg. in full-grown specimens. This indicates that, though there is a considerable enlargement and branching in the intestinal tract, nevertheless the interior surface behaves as if it were the surface of a proportionally growing body, controlling growth in the way mentioned above. Further experiments have been done in determining quantitatively *starvation, temperature influence, metabolism.* In higher temperatures, occasional *monsters* and *duplicitates*, resembling the results of experimental injuries, are found among hatching animals. Full descriptions of methods and results will be given in another place.

Probable absence of a chromatophore activator in Limulus polyphemus. Ruth Snyder Cooper.

Hosoi (1934) reported a chromatophore activator in several organs of *Penaeus*; furthermore, Hanström (1937) found that heads, but not eyestalks, of *Hippa* and *Gebia*, and heads and thoraces of certain insects possess chromatophore activators. It therefore seemed of interest to test organs of the arachnoid *Limulus* for a chromatophore activator.

Sea water extracts were made of lateral eyes, plus some adherent nerve fibers, removed from living adult *Limulus*. Cephalothoracic and abdominal injections of such extracts into black-adapted, white-adapted, and blinded *Palaemonetes* induced no chromatophoric contractions. Survival being highest following abdominal injections, all subsequent injections were abdominal.

After killing adult *Limulus* in 80° C. sea water (5 minutes), organs were immediately removed and stored at 4.5° C. until extraction. Diluted blood (1 : 1.66 sea water) of such freshly killed *Limulus* effected no chromatophoric responses of black-adapted or blinded *Palaemonetes*; undiluted blood was lethal. Similar organs of ten *Limulus* were mashed together and extracted in at most $2\frac{1}{2}$ cc. of sea water at 100° C. for ten minutes. Filtered extracts of "brains," thoracic plus abdominal nerve cords, portions of male gonad and gastro-hepatic glands, and nephridia were injected into black-adapted, white-adapted, and blinded *Palaemonetes*. Survival of *Palaemonetes* after injection was as follows: for eye—100 per cent, gonad plus gastro-hepatic gland—100 per cent, nephridia—66 per cent, blood—66 per cent, nerve cord—55 per cent, and brain—20 per cent. All surviving animals failed to show chromatophoric responses.

The experiments were controlled by sea water and *Palaemonetes* eyestalk extract injections into *Palaemonetes*, as well as by tests of adaptability to white or black backgrounds. Although but 50 test *Palaemonetes* were employed, it appears that the above organs of *Limulus* are devoid of a chromatophore-activating principle.

Intracellular pH studies on the ova of Mactra solidissima. C. G. Grand.

The germinal vesicle of the *Mactra* egg occupies a large part of the cell. It persists for hours and does not break down normally until after insemination of the egg.

The intracellular pH and acid of injury of *Mactra* eggs in the germinal vesicle stage were determined by the micrurgical technique. The determinations of pH were made colorimetrically using 0.4 per cent aqueous solutions of phenol sulphon-phthalein indicators. The indicators used were: cresol red, phenol red, brom cresol purple, chlor phenol red and brom cresol green.

None of these indicators served as vital stains in ordinary immersion methods except when the cells were dead. Dead cells stain intensively with most of these indicators, the pH reaction of the cytolyzed material being the same as that of sea water. This fact made it possible to determine those cases in which the microinjections were performed without injury.

The eggs were operated upon while immersed either in normal alkaline (pH 8.4) or acidified (pH 5.6) sea water. This procedure was used to check against the possibility that cytoplasmic pH was influenced by the surrounding sea water.

Cresol red, phenol red and brom cresol purple were injected into the cytoplasm of the eggs. The diffuse coloration resulting from the injections was as follows: cresol red—*yellow*, phenol red—*yellow orange* and brom cresol purple—*blue*. Similar injections into the germinal vesicle gave the following colors: cresol red—*pinkish yellow* (near its acid range) and phenol red—*red* (alkaline).

The action of fixatives on the isolated cell nucleus (Film). W. R. Duryee.

When a nucleus, such as the germinal vesicle of an amphibian egg, has been divested of its coverings of cell wall and cytoplasm, it becomes especially suitable for the most precise observations. The film records the process of normal nuclear fixation with Bouin's, Zenker's and Carnoy's fluids, showing that in each case the hydrogen ions penetrate first, reversing the sign of the charge on the nuclear colloids, and that the other components enter later, causing secondary precipitations, displacement of the chromosomes, and shrinkage of 60 to 79 per cent in volume. It is demonstrated that gelatinous material is extruded through the nuclear membrane during shrinkage. Such material may give rise to the "shrinkage space" seen in sections of ovocytes and should not be confused with cytoplasm.

Superimposing Zenker's fluid on Carnoy's produces an entirely different picture from the latter alone. Consequently the fixed image is neither permanent nor unalterable.

Stretching, tearing and dislocation of the chromosomes are shown. Of particular significance is the fact that the swelling of the colloidal matrix due to the primary penetration of hydrogen ions may result in liquefaction, allowing the chromosomes to sink to the inner surface of the nuclear membrane before secondary coagulation sets in. Such chromosomes are unnoticeable in sectioned material.

Weak fixation with 0.001 molar $CaCl_2$ is reversible upon addition of 0.003 molar NaOH. Successive reversals with increasing strengths of acids and bases are demonstrated.

It is concluded that none of these fixatives can yield a valid picture of the nucleus.

The melanophore-expanding activity of the ascidian neural gland. Adolph Elwyn.

Tests conducted with the neural glands of *Molgula manhattensis* and *Molgula occidentalis* definitely indicate the presence of a substance capable of effecting pigmentary changes. Saline suspensions of pulverized acetone-dried glands of *M. manhattensis* when injected into the anterior lymph sac of *Rana pipiens* always gave positive reactions. The pale or partly pale frogs began to darken in about thirty or forty minutes, reached a certain maximum which was maintained for a variable period and slowly regained their initial color value, the whole reaction lasting between two and four hours. Injection of similar quantities of other *Molgula* tissue or of normal salt solution produced no observable changes. The introduction of twenty-five freshly mashed glands likewise produced a noticeable darkening though not as marked as with the dried material, which was used in larger quantities.

Most striking was the effect in tadpoles of *Rana catesbiana*. In four tests all the animals injected with dried gland suspensions showed intense darkening, beginning in a few minutes and lasting a variable time, one over five hours, the others from two to four hours. Again the injection of other *Molgula* tissue produced no appreciable change.

In *Fundulus heteroclitus* the changes were much more irregular and difficult to interpret. In four cases there was only a local darkening varying in extent, in the region of injection, often supplemented by the appearance of small dark patches in other regions of the body. These results had to be completely discounted since similar changes could be produced by other *Molgula* tissue or even by normal saline alone. Only in three experiments were the results positive, the local reaction being gradually followed by a uniform darkening of the whole body, which was maintained for several hours. Further tests will have to be made.

Positive results were likewise obtained from the freshly dried neural glands of the larger Florida form, *Molgula occidentalis*, collected in 1937. The test animals were *Rana pipiens*, *Anolis carolinensis* and *Hyla cinerea*. It was found, however, that glands which had been kept in the dry condition for four months or longer had apparently lost their capacity for producing pigmentary changes.

Cardiac rhythm in Pecten irradians (Lamarck). Richard W. Foster, John D. Crawford and Albert E. Navez.

The frequency of heart-beat of *Pecten irradians*, for the heart in situ and excised, has been studied in relation to its autonomism and its dependence on other organs.

In situ the frequency is determined by some characteristic proper to each individual heart, which can be modified (mostly reduced even to complete block) by movements of the valves, gills or parts of the mantle.

The absolute value of the rate depends on the size of the animal among other factors. A 5-cm. *Pecten* under optimum conditions has a heart rate of 35 beats per minute at 22–23°.

The excised heart has a frequency of contraction decidedly lower than the in situ heart, depending on the portions of the heart excised.

Its frequency may range from about 27 beats per minute to values as low as 8 beats per minute. It is very sensitive to water circulation, aeration, temperature and stimulation, the amplitude of the beat being specially sensitive to temperature.

The nucleus, when shelled out by tearing the egg into acidified sea-water containing phenol red, first turned red (alkaline) and then shifted rapidly to the yellow color (acid) of the surrounding medium.

Mechanically injuring the eggs produced an acid of injury, the pH of which was determined by observing the color changes of previously injected indicators. Before cytolysis, brom cresol purple was blue (alkaline) and phenol red was yellow (acid), representing a pH of approximately 6.8. Immediately following cytolysis these indicators changed to the color of their acid range, viz., yellow. The lower pH range of the acid of injury was determined by using brom cresol green which gave the blue of its alkaline range.

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In conclusion, the pH of *Mactra* eggs was found to be as follows: Cytoplasm, 6.8 ± 0.2; nucleus (germinal vesicle), 7.4 ± 0.2. Mechanical injury with cytolysis gave the acid of injury of pH 5.4 ± 0.2. These findings are in accordance with those of other investigators who obtained similar pH values from studies on other types of cells.

The alarm reaction and adaptation syndrome in lower vertebrates (Fundulus majalis). Stephen Karády.

When adult *Fundulus majalis* and *heteroclitus* are given intraperitoneal injections of sublethal doses of various injurious agents (histamine, adrenalin, pituitrin, atropine), their reaction to the first dose is always the same, being independent of the nature of the drug. The syndrome consists of haemorrhages into the intestinal tract, hypertrophy of the islands of Langerhans, fatty degeneration of the liver, etc. Such changes constitute the response of the organism to injury as such, and can be termed the alarm reaction.

These pathological changes disappear in 24–48 hours. Thereafter the resistance of the fish is increased, not only to the same drug which produced the reaction but also to the others as well. The fish can now tolerate doses which ordinarily are lethal. In like manner the color changes produced in the skin are less intense after the first injection. This condition is called the stage of non-specific resistance.

If the treatment with any one of these drugs is continued for a longer time (8–10 days) the resistance of the organisms to that particular agent increases continuously, no signs of the alarm reaction appearing. But the resistance to the drugs other than the one used disappears (stage of specific resistance).

If the treatment is still further continued, the specific resistance acquired during this stage is gradually lost, and the fish die with symptoms similar to those observed during the first stage. This last condition is called the stage of exhaustion.

Micro-estimation of protein adsorption at oil-protoplasm interfaces. M. J. Kopac.

The adsorption of proteins at oil-protoplasm interfaces has been demonstrated by studying the Devaux effect [Kopac, this number]. Frequently a spontaneous Devaux effect cannot be obtained on oil-drops injected into starfish eggs several minutes after cytolysis. A method has been developed for showing that protein adsorption, nevertheless, occurs under these conditions.

If continuity between an oil-drop and a micropipette is maintained, the oil may be slowly drawn back into the pipette. The area of the interface is reduced by decreasing the diameter of the oil-drop. Where the oil is in contact with aqueous phases lacking protein, the diminishing oil-drop remains perfectly spherical until it is entirely drawn into the micropipette. If, on the other hand, the oil has been in contact with an aqueous phase containing protein, and then slowly aspirated into the micropipette, the sphericity is maintained only to a critical diameter. A further decrease in diameter causes the appearance of the Devaux effect. The wrinkling which occurs at this critical diameter, according to the studies of Devaux and Langmuir, is due to a monolayer of protein completely covering the oil at the interface. The adsorbed protein is concentrated as the area of the interface is decreased until the monolayer coated condition obtains.

The ratio, δ, which equals $\pi d_c^2/\pi d_o^2$, may be used as an index to indicate the amount of protein adsorption. Here d_o represents the original diameter of an oil-drop in contact with the aqueous phase and d_c is the critical diameter at which the Devaux effect appears. If $\delta = 0$, no adsorption has occurred. If $\delta = 1$, the Devaux effect appears spontaneously. With $\delta < 1$, protein adsorption is indicated, the amount of adsorption being directly proportional to the value of δ. In starfish oocytes cytolyzed for a few minutes, the δ-values are less than 1 but, in many cases, the values approach 0.9, indicating that appreciable amounts of protein have been adsorbed although insufficient to produce a monolayer coating on the original drops.

Accumulative adsorption of protein can be demonstrated by expanding the same oil drop and then decreasing its diameter. If the new critical diameter is the same as before, no additional adsorption occurred. However, an increase in critical diameter indicates accumulative adsorption. Accordingly, rates of adsorption may be obtained by combining critical diameter measurements with time.

Extremely small amounts of protein can be estimated. For example, an oil-drop, 10 microns in diameter, has a surface area of 3.14×10^{-6} cm^2. By taking Philippi's value of 1.15 m^2/mg. for egg albumin, a monolayer of protein at such surface would correspond to 3×10^{-13} g. On the basis of a molecular weight of 35,000, this weight of protein represents approximately 5×10^6 molecules.

Effect of the vitelline membrane on coalescence of Arbacia eggs with oil-drops. M. J. Kopac and Robert Chambers.

Various marine eggs, if denuded of their jelly layer and tough extraneous membranes, will coalesce with oil-drops [*J. Cell. Comp. Physiol.*, 9: 331; 345, 1937]. An oil-drop flowing from a micropipette and in contact with the cell-surface is expanded at a constant rate until coalescence occurs. There is a critical diameter (d) of the oil-drop below which the cell will not coalesce with the oil. Coalescence does occur if the diameter exceeds the critical value. The resulting potential energy change (ΔE) is approximately equal to the surface area (πd^2) of the drop times the difference in tension (ΔT) at the surface of the drop before and after coalescence, namely, $\Delta E = \pi d^2 \Delta T$.

The critical diameter and accordingly the ΔE indicate the presence of a potential hill at the cell-surface. By determining the critical diameter of a coalescing oil-drop it is possible to calculate the magnitude of the potential hill. This value may be used as an index to denote the tangential rigidity, inter alia, of the cell-surface. In these experiments the potential hill of eggs under experimental treatment was compared with that of normal, unfertilized eggs, and this ratio is represented by δ in the table.

Cottonseed oil with a ΔT-value of approximately 10 dynes per cm. was used. The *urea-eggs* were unfertilized eggs immersed in 1 M urea for 2 minutes and returned to sea water. The fertilization membranes were removed from inseminated eggs by shaking. The critical diameters, d, are the most probable values under a given experimental treatment while the d_o values are those of oil-drops with which normal, unfertilized eggs coalesced.

Treatment of Eggs	Age of Eggs Hours	Critical Diameters		$\delta = \frac{\pi d^2 \Delta T}{\pi d_o^2 \Delta T} = \frac{d^2}{d_o^2}$
		d_o	d	
Control	1–2	97 μ	97 μ	1.00
Fertilized, membranes removed	<1	77	23	0.09
	<1	92	25	0.07
Immersed in 1 M urea, 2 minutes	<2	97	32	0.11
	4	97	37	0.15
	7–8	82	28	0.12
Churned with microneedles	2	97	40	0.17
	8	82	35	0.18
Aged in sea water	8	97	82	0.71
	9–10		62	0.42
	12		44	0.20
	15–16		26	0.07
	20		9	0.01

Fertilized eggs yield low δ-values. In these eggs the vitelline membrane has been transformed following insemination to a fertilization membrane and subsequently removed by shaking. This result shows that the vitelline membrane is a significant barrier (high potential hill) to coalescence. Eggs which were churned and eggs treated with urea (no fertilization membrane develops following insemination) have correspondingly low δ-values. Here the coalescence data indicate that the vitelline membrane is either mechanically destroyed or chemically dispersed. Likewise, on the basis of decreasing δ-values, one concludes that the vitelline membrane gradually disintegrates in aging, unfertilized *Arbacia* eggs.

The osmotic pressure of gum acacia solutions. George Saslow.

In 1934 Dodds and Haines reported that the osmotic pressure of 6 per cent acacia solutions was reduced by 0.9 per cent NaCl to a value about half the colloid osmotic pressure of mammalian serum. The successful use of acacia solutions in total plasmapheresis of dogs (Amberson and co-workers) and measurements of the osmotic pressure of acacia solutions used in frog perfusions (Saslow) suggested that the measurements of Dodds and Haines be repeated.

The osmotic pressures were measured with the Krogh and Nakazawa osmometer; a precision of -8 mm. H_2O in 250 mm. was attainable. Three different samples of acacia were used. All were preparations of the Lilly Research Laboratories.

The results were as follows. NaCl added to 6 per cent acacia reduces the colloid osmotic pressure, the more so the greater the final salt concentration. The colloid osmotic pressure of the three samples, in 0.9 per cent NaCl, was 262, 250 and 255 mm. H_2O at 20°. The osmotic pressure of one sample in buffered Ringer-Locke's was 248 mm. The measurements agree well with those of others on material from the same source (Wies and Peters, Turner).

Since the colloid osmotic pressure of human serum containing 6.5 per cent protein was found to be 256 mm. H_2O (Wies and Peters), it is clear that the conclusions of Dodds and Haines do not apply to acacia prepared according to the procedure of the Lilly Research Laboratories.

Differences between Scottish and American amebas of the species Chaos chaos Müller. A. A. Schaeffer.

The accounts describing nuclear division in the common laboratory ameba, *Chaos diffluens*, vary widely, as is well known. Thus, for example, Taylor, Carter and their co-workers in Scotland report the typical occurrence of an irregular type of mitosis that might perhaps be better described as amitosis; but American investigators, on the other hand, report division as definitely mitotic. On the assumption that there is no genetic difference between the Scottish and the American amebas, the diversity in the accounts setting forth the details of nuclear division has in consequence been laid to faulty technique or to the use of pathological material. It appears now, however, that the reason for this conclusion is more complicated and may even be unjustified.

A culture of amebas obtained through the kindness of Monica Taylor of Dowanhill, Glasgow, Scotland has been cultured in Woods Hole with the same culture methods as are used to grow American amebas, and a preliminary survey of the characteristics of the two strains shows that the *Chaos diffluens* of Scotland is distinctly and consistently different from that of America.

The main differences are as follows:

(1) The Scottish amebas have fewer ectoplasmic ridges than the American amebas.

(2) The cytoplasmic division stage—moruloid—is typically a freely rolling ball; the American ameba at this stage is firmly attached to the substrate.

(3) Nearly all nuclei of the Scottish amebas are irregularly folded; this condition is extremely rare in the American strain.

(4) If the Scottish amebas are agitated when in that part of the moruloid stage corresponding to late anaphase, a very distinctive shape is taken on by the ameba. The main mass of protoplasm is a smooth sphere or ovoid with a tuft of pseudopods at either end; the American ameba does not respond to agitation in this way.

(5) Only a few permanent preparations of nuclear division stages have been made, but these seem to bear out Taylor's conclusion that mitosis is very irregular.

(6) The Scottish amebas grow as fast, if not faster than the American strain, under the same cultural conditions and appear to be quite as hardy.

There has been no opportunity so far to observe the formation of agametes which Taylor describes.

These two strains undoubtedly belong to the same taxonomic species so far as the general morphology of the vegetative stage is concerned. The difference between the two strains may be genetical or it may be due to a submicroscopic parasite or symbiont. Only further work can supply the answer.

The Scottish strain is apparently not confined to Scotland, for this strain or variants of it, have been reported from other countries of Europe by various investigators since 1867, when von Stein first described a multinucleate *Chaos diffluens.*

Induction in Griffithsia. Victor Schechter.

In following up work done several years ago on the effect of electric current (*Jour. Gen. Physiol.*, **18**, no. 1) and the centrifuge (*Biol Bull.*, **68**, no. 2) on the induction of shoots and rhizoids in *Griffithsia* cells, a preliminary mapping has been made of the effects of butyric acid, indolebutyric acid and a-naphthalene acetic acid. The use of hypertonic sea water and butyric acid as in artificial parthenogenesis of egg cells has been largely lethal in effect but, when the cells survived, a greater number of shoots were induced. When material was allowed to develop in 0.4 per cent and 0.04 per cent sea water solutions of N/10 butyric acid, a stimulation in number of shoots formed was indicated. a-Naphthalene acetic acid showed a stimulating effect on shoots in 0.00001

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EDITOR'S NOTE

Through an error, the title of the abstract by A. A. Schaeffer on p. 374 of the October issue was incorrectly printed. The correct title should be: "Differences between Scottish and American amebas of the species *Chaos diffluens* Müller," not "*Chaos chaos* Müller."

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The effect of sodium azide on the respiration of frog muscle. J. N. Stannard.

The results of the present investigation indicate an essential discontinuity in the action of sodium azide on the respiration of resting frog muscle compared with muscle whose oxygen consumption has been forced to higher levels by one of several means.

Under basal, resting conditions (pH 7.38) the respiration is not inhibited by moderate concentrations (M/1000) of azide but instead a stimulation (50–75 *per cent*) occurs. Higher concentrations lead to gradual inhibition after an hour or more. In contrast to the effect on the resting metabolism the increment in respiration caused by the presence of caffeine (0.04 per cent) or isotonic KCl is almost completely eliminated within ten minutes by M/1000 azide. The respiration then remains at the resting level (i.e. the "resting-azide" level) for several hours. The oxidation of para-phenylenediamine is largely, but not completely, inhibited under these conditions, but the increased oxygen consumption due to a carrier such as pyocyanin is not eliminated.

In addition sodium azide inhibits or greatly reduces the burning of carbon monoxide characteristic of resting frog muscle without further effect on the basal oxygen consumption (i.e. the "resting-azide" level persists). In this respect the action of sodium azide differs from that of cyanide, since KCN, over a wide range of concentrations, reduces the resting oxygen consumption and the increment due to carbon monoxide equally.

The effect of azide on frog muscle is, unlike its action on yeast (Keilin), little changed by variations in pH. Even at pH 4.6, although the stimulating action of M/1000 azide does not occur, the rate of inhibition of the resting respiration is only slightly increased. Furthermore, inhibition of the fractions due to caffeine, KCl, etc. takes place readily at pH 7.38.

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These results are tentative and together with effects on rhizoids must be quantitatively evaluated.

Bacteria in relation to longevity of egg cells. Victor Schechter.

In a study of the longevity of egg cells the influence of bacteria must be taken into account. It has been found that paper-filtered sea water in which *Mactra* eggs have aged and died is extremely toxic to fresh eggs. Toxicity may be in good part removed by pasteurization at 60–80° C. or by Berkefeld filtration. These methods do not show conclusively whether bacteria themselves, or whether large colloidal particles of disintegrated eggs, are responsible for the lethal effect; the Berkefeld filter would remove larger colloidal particles as well as living bacteria and heat would denature proteinaceous toxins as well as kill bacteria. The use of the bactericide "Merthiolate" seems to have a beneficial influence if added early to an egg culture but none on fresh egg cells added to an aged culture. This may prove, if substantiated, that egg disintegration products kill other eggs and that the action of bacteria is not directly on living eggs. The results so far are clouded, however, because "Merthiolate" is itself toxic to eggs and the working differential is small. Bacterial counts in low calcium solutions, which prolong the life of the eggs, show no differences which can be correlated with the effects on longevity.

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Respiratory stimulants and gastrulation in Arbacia. A. J. Waterman.

With the purpose of determining the effects, if any, of artificially increased respiratory rate and cleavage retardation on gastrulation in *Arbacia*, the early blastula stage was placed in graded concentrations of 2,4-dinitrophenol, 3,5-dinitro-o-cresol, iodoacetic acid, pyocyanine and methylene blue. The cultures were examined 17–48 hours later and cases of developmental arrest were transferred to fresh sea water.

The results are proportional to the concentration and to the length of exposure. All produce inhibition, retardation and abnormalities in high concentrations and some are possibly very slightly stimulating in weak concentrations. Effective concentrations are different for each reagent. 3,5-dinitro-o-cresol is most toxic and iodoacetic acid least. In sea water inhibited blastulae may form large ectoblastulae (animalization) usually with skeleton; large globular types with skeleton and relatively undifferentiated gut or small endodermal mass; abnormal plutei especially as regards skeleton and arm differentiation, number and size; or typical plutei of small size. There is considerable variation depending upon susceptibility.

The absence of recognizable endoderm does not prevent the formation of skeletal material or the differentiation of ectoderm and even the pluteus body shape. However, the arms never develop. Skeletal modifications include, asymmetry, accessory projections and more extreme modifications. Gastrulation and differentiation are not affected directly by a retarded cleavage rate during the experimental period, nor is gastrulation stimulated. Concentrations strong enough to affect gastrulation either retard or inhibit it. In sublethal concentrations gastrulation may be inhibited for 17–32 hours at least without effect on the capacity to gastrulate in a typical manner. In only a few cases were occasional exogastrulae formed and these did not differentiate. In concentrations of certain of the reagents which possibly stimulated later development, the embryos did not survive as long as the controls.

Does transfer of pronuclei ever occur in conjugation of Paramecium caudatum? Ralph Wichterman.

In an earlier account (*Biol. Bull.*, Oct., 1937) a method was described for studying the activities and sequences of nuclear events during conjugation. A pair of conjugants is isolated and placed in a micro-compression chamber where the organisms are temporarily prevented from spiralling while observations are made with the microscope. Additional information is given here.

Diagrams of conjugation of *Paramecium caudatum* in textbooks show a breakdown of the contiguous membranes shortly after the union of the conjugants. This breakdown of the membranes would lead one strongly to suspect that there is a cytoplasmic transfer. In a study of the living animals such a transfer has not been observed while the conjugants are fused along their oral grooves. Their individual membranes may be seen intact at this stage. Indeed, active cyclosis in each conjugant occurs as it does in the vegetative stage. The micronuclear products as well as the granules and crystals are carried about in the streaming protoplasm.

At the beginning of conjugation, there is a progressive dedifferentiation of the ingestatory structures which appear to pass into a sol state.

After the conjugants separate along the oral groove at the completion of conjugation, a thin clear elastic strand is the last structure to hold the organisms together. This strand, which finally breaks, consists of the stretched and fused apices of the paroral cones.

Calkins and Cull (1907) described chromatin elimination in the second and third "maturation" divisions but stated that it did not occur in the first division. I find it to be very conspicuous in this first division where the long telophasic separation spindle between the daughter nuclei is discarded into the cytoplasm and left to degenerate.

One is struck by the nuclear aberrations especially after the first pre-zygotic division. Also, there is considerable non-synchronous nuclear activity in both conjugants of a pair, i.e. one conjugant in a pair may show a nuclear stage more advanced than its co-conjugant.

Evidence does not indicate a transfer of pronuclei from one conjugant to another.

More complete data on time relationships with photographs of all the important living stages of conjugation will be presented in the full paper. Motion pictures of all three pre-zygotic divisions are in the process of being made.

Studies in calcification. IV. A contribution to the problem of skeletal calcification in the teleost, Fundulus heteroclitus. E. Alfred Wolf and Alfred S. Levenson.

The Gömöri modification of the silver nitrate method was applied to the study of skeletal calcification in the teleost fish, *Fundulus heteroclitus*, mainly in the adult stage and to a smaller extent in young fish of nine days after hatching. The tissues investigated were: calcified cartilage and membrane bones of the skull, vertebrae, ribs and the so-called "fish bones." In the case of calcified cartilage, the usual staining methods give no safe criterion for the extent of the penetration of calcium salts into the cartilage. The claim that the hypertrophied appearance of the cells in the cartilage is an indicator of those regions where calcium is precipitated was shown not to hold. No relation could be found between the presence of this type of cells in the cartilage and the deposition of calcium as revealed by the silver method. The perichondrial sheath surrounding the cartilage consists of a modified transparent type of fibrous tissue within which calcium is assumed to be deposited. The silver nitrate method applied here gives positive proof of the presence of calcium in this type of tissue. No calcium deposits are present wherever the fibers have not undergone this transformation into the transparent type. Two types of cartilaginous bones were found: in one type the calcium deposits have penetrated from the perichondrium into the cartilaginous parts of the bone; in another type the calcification is confined to the perichondrial sheath. The calcified perichondrium is, in structure, identical with the membrane bones. Vertebrae have a cartilaginous origin, but the cartilage calcifies at a very early stage and can therefore not contribute any essential part to the structure of the fully grown vertebrae of the adult. Ribs maintain their cartilaginous core though they are completely enclosed by the calcified perichondrium.

Mitotic activity of the islands of Langerhans and parathyroids of rats following pituitary extract and colchicine injections.[1] Opal M. Wolf.

The mitotic activity of the endocrine glands of rats following daily injections of an [2] alkaline extract of the anterior lobe of the pituitary gland at 1-cc. and 2-cc. levels has been studied. Control and pituitary-injected animals were etherized the morning following the second, fourth, sixth and tenth days of injection. Nine to fifteen hours before the male animals were etherized 0.3 mg. (per 100 grams of body weight) of [3] colchicine dissolved in distilled water was injected subcutaneously. At autopsy tissues were fixed in Bouins', dehydrated by the anilin oil-dioxan method, cut at 10 micra and stained in Delafield's or Heidenhain's haematoxylin.

By means of an ocular micrometer it was estimated that nine islands of Langerhans in a control rat, 186 grams, averaged 10.5 mitoses per 1,000 cells. The average number of four controls was 7.45. The pituitary-injected rats showed the following counts per 1,000 cells.

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[1] The experimental work was performed at Goucher College. I am indebted to Miss Naomi Jaffe for technical assistance. Part of the sections were studied at the Marine Biological Laboratory.

[2] The growth extract was purchased from E. R. Squibb and Sons.

[3] Colchicine U. S. P. was purchased from Merck and Co. in the form of a powder.

	1-cc. Level	2-cc. Level
8 days	15.48	21.26
6 days	—	20.90
10 days	8.89 7.45	21.13

Mitoses were counted in five pituitary-injected and three control female rats killed 16 to 23.5 hours after colchicine injection on the fourth day of the experiment.

Hours after Colchicine Injection	Controls	1-cc. Level	2-cc. Level
16.	6.2	23.25	17.28
20.5			27.55
21.		22.81	
21.5		26.12	
23.	4.17		
23.5	5.19		

The average count of the three largest sections of the parathyroid from control male rats showed 2 to 7 cells in mitosis (one control showed 32 cells). The greatest number, 47 mitoses, occurred in a male on the 2-cc. level killed the tenth day of injection. A female killed the fourth day had 57 mitoses on the 1-cc. level for the highest count and another 70 on the 2-cc. level. The highest count for the female controls was 14 mitoses.

Oviducts of pituitary stimulated females, Rana pipiens. Opal M. Wolf.

In an earlier work Wolf (1928) reported that castration caused an involution of the oviducts of female *Rana pipiens* and injections of a water-soluble extract of mammalian ovarian hormone caused an hypertrophy which exceeded the size of the oviducts of normal control animals. It seemed of interest to study the effects of sub-cutaneous injections of bull-frog anterior lobe of the pituitary on the ovary and oviducts of mature female frogs, *Rana pipiens*, during the summer months following the breeding period (having reported, 1937, that isolated adult males in July shed sperm, spermatogenesis occurred, and the thumb-pads on the first finger of the fore limbs became ridged during pituitary injections).

From July 24 to August 17, a total of 15 anterior lobes of the pituitary gland of *Bufo catesbiana* were injected into each adult female *Rana pipiens*, approximately 8.5 cm. in length, tip of mouth to anus. At the end of the period the animals were etherized and the ovaries and oviducts of experimental and control animals were weighed. Tissues were fixed in Bouins' fluid. Histological preparations were made of parts of the ovary and of the oviduct 1.5 cm. from the uterus.

The average weight of the ovaries of injected animals exceeded that of the controls by 62 per cent; the oviducts of the treated animals were 132 per cent more than the controls. Ovulation did not occur but in the experimental animals the nuclei of the larger ova were eccentric or very near the surface. The controls showed centrally placed nuclei. (Moore, 1937, reported that the nuclei of pituitary stimulated ova moved to the surface.) The oviducts of the injected females showed hypertrophied albumen-secreting cells. In relation to the earlier work, it appears that the hypertrophy of the oviducts of the experimental animals is due to an increased output of ovarian hormone from the pituitary-stimulated ovaries.

The effects of Roentgen irradiation on cleavage and early development in the annelid, Chaetopterus pergamentaceus. R. A. Young.

The eggs of *Chaetopterus* were exposed to 7800r per minute for varying lengths of time and under varying conditions in order to compare the direct and indirect effects of Roentgen irradiation. The results for exposures of several minutes are as follows. Eggs irradiated free in the sea water showed a slight acceleration in the rate of first cleavage, followed by a gradual decrease in the rate of development. Eggs irradiated in the presence of pieces of egg sac showed no significant change in rate of first cleavage, but the later development was slower and slightly abnormal. Eggs irradiated in the egg sacs or in the intact animal showed marked delay in first cleavage and greater abnormalities in larval stages.

The crucial experiment to test the indirect influence of Roentgen irradiation on cells was performed in the following manner. Part of a *Chaetopterus* was shielded during irradiation and eggs were removed from that region immediately after the exposure and inseminated. These eggs showed no radiological effects on their cleavage or later development. But eggs removed from the same region sometime later showed effects quite similar to those shown by eggs taken from the irradiated area. In other words—the effects of irradiation had spread beyond the region directly exposed.

Normally these eggs remain in the germinal vesicle stage while in the egg sacs where the pH is 6.9. The germinal vesicle breaks down after twelve to fifteen minutes in sea water (pH 8.2). Experiments were made to ascertain the influence of the hydrogen ion concentration of the medium surrounding the eggs at the time of irradiation. The results indicate that the more marked response which is shown when eggs are exposed in the egg sacs or in the intact organism is not due to change in pH over this range.

The effect of perisarc removal on regeneration in Tubularia crocea. E. Zwilling.

Several experiments on Tubularia reported by Dr. Barth [1] indicated that a glass capillary tube placed over an end otherwise free to regenerate would inhibit regeneration. In addition, Dr. Barth observed that if the coenosarc at such a free end was retracted into the perisarc, regeneration never occurred. An outgrowth of this work is an experiment in which the perisarc is removed somewhere along the length of the stem and the coenosarc exposed to freely circulating sea water. A number of experiments were performed to determine some of the relationships involved in the regeneration of coenosarc thus exposed. It was found that hydranths do form at the exposed region and that, as a rule, there are two of these and that they are symmetrical mirror-images of each other; that the rate (vol./time) of formation of these hydranths is directly proportional to the amount of coenosarc exposed. In one group of older stems it was found that the morphology of the regenerants varied with the area exposed, namely: with smaller areas small single hydranths formed, with larger exposed areas double hydranths were formed with their distal ends more or less fused. With very large areas two fully-formed hydranths in contact at the oral ends were formed. Laceration of the coenosarc is not responsible for regeneration since exposure of non-lacerated and lacerated coenosarc gave essentially the same results. Further—when the coenosarc is lacerated and the perisarc is replaced no regeneration occurs. It was found that the free ends, especially distal, exert considerable dominance over the exposed region. The greatest number of regenerants was obtained when both ends were ligatured and the exposed region was removed from their influence. An exposed area near the distal end of the stem will completely inhibit the regeneration of a more proximally placed exposed area. It is suggested, in the light of the work of Barth and of Miller (unpublished), that the exposure of the coenosarc tissue to a high oxygen tension is the important factor involved in the stimulation of regeneration.

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[1] Barth, L. G., '38, *Physiol. Zoöl.*, **11**: 179.

Vol. LXXV, No. 3 December, 1938

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE TRANSPORT OF CARBON DIOXIDE BY ERYTHROCYTES AND PLASMA IN DOGFISH BLOOD

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During the summer of 1936, unsuccessful attempts were made by J. K. W. F. and J. R. P. to measure (using the chemical method of Ferguson and Roughton, 1934) the carbamino compounds of CO_2 with hemoglobin of mackerel blood and dogfish blood. The difficulty of preparing stable solutions of hemoglobin from these bloods proved to be the most serious obstacle. An attempt was then made to apply the kinetic method of Meldrum and Roughton (1933) to dogfish blood. This too was unsuccessful because carbonic anhydrase could not be completely inactivated by cyanide as required in this method.

Some evidence as to the state of CO_2 in dogfish blood was then sought by determining the distribution of CO_2 between the erythrocytes and plasma. A few preliminary experiments revealed, unexpectedly, that the erythrocytes seemed often to contain more CO_2 than the plasma, a relationship which is the opposite to that found in mammalian blood. A continuation of these experiments, during the summer of 1937, has established the fact that there are peculiarities in the transport of CO_2 in dogfish blood, which may be of general significance.

METHODS

Dogfish were used exclusively in this study as they were about the only available source of sufficient amounts of blood. Thirty of the thirty-two fish used were of the smooth variety (*Mustelus canis*). The other two were spiny dogfish (*Squalus acanthias*). Usually the blood was drawn by paracentesis of the dorsal blood vessels through the ventral body wall behind the cloaca. Aeration of the gills could be maintained throughout this procedure while the fish was held almost motionless. Heparin (15 units per cc. of blood) proved to be a satisfactory anticoagulant, but defibrination was just as satisfactory for most purposes. At first, attempts were made to use fluoride and oxalate (the fluoride to check glycolysis). It soon appeared that both fluoride and oxalate caused progressive swelling and eventual hemolysis of the erythrocytes. This phenomena is being investigated further. Fortunately the use of fluoride proved unnecessary. When the blood was kept at 10° C., the loss in CO_2 capacity at a CO_2 pressure of about 20 mm. Hg was less than 1 vol. per cent after eight hours. Each dissociation curve was completed in less time than that.

Equilibration of the blood with gas mixtures was done by the first method of Austin et al (1922) in a water bath at a temperature of 22° C., or sometimes of 23° C. The equilibrated blood was segregated in a centrifuge tube joined to the tonometer by wide rubber tubing. The gas phase was analyzed in either a Van Slyke or a Haldane apparatus. After samples of whole blood had been removed for the various analyses (O_2, CO_2, H_2O and Cl) and the determination of cell volume, the remainder was centrifuged under oil at about 2800 r.p.m. for 30 minutes. Analyses were then made on the plasma. When the volume of packed cells was sufficient, analyses were made on them, frequently for chloride and H_2O, but only on one occasion for CO_2.

CALCULATIONS

The concentration of CO_2 in the erythrocytes was calculated by the formula

$$C = \frac{B - V \times P}{v},$$

where $C = CO_2$ in erythrocytes (v.p.c.), $B = CO_2$ in whole blood (v.p.c.), $P = CO_2$ in plasma (v.p.c.), $V =$ volume of plasma per cc. whole blood, $v =$ volume of erythrocytes per cc. whole blood. In all cases in this paper CO_2 means total CO_2.

The distribution ratio of CO_2 between cells and plasma was calculated by the formula:

$$rCO_2 = \frac{C}{P} \times \frac{Wp}{Wc},$$

where $Wp = gH_2O/100$ cc. plasma, $Wc = gH_2O/100$ cc. cells.

The distribution ratio of chloride (rCl) was calculated in a similar manner.

SOURCES OF ERROR

An analytical figure for CO_2 in plasma or blood may be in error by about 0.5 v.p.c. When the CO_2 content of the blood is about 10 v.p.c. (a typical value for venous dogfish blood), such an error might produce a large error in the calculated value of *C* and hence of rCO_2. For example, if the analysis of the plasma gave a result too high by 0.5 v.p.c., and the analysis of the whole blood too low by 0.5 v.p.c., an error of 38 p.c. would result in rCO_2. For this reason little importance has been attached to individual values of rCO_2. Each value of rCO_2 reported in this paper is a mean for a group of comparable determinations.

In addition to random errors, a systematic error may be inherent in the hematocrit determinations since centrifuge speeds above 3000 r.p.m. were not used. If the hematocrit numbers are systematically too high, due to failure to separate completely the plasma from the cells, the error would tend to obscure any real discrepancy between the concentrations of CO_2 in cells and plasma. Similarly in the estimation of chloride and water content of the cells, allowance for plasma trapped between the cells would tend to diminish the reported values of rCl and increase those of rCO_2. Thus in every case the systematic errors, if they are significant, would tend to obscure, not enhance, the discrepancies which are the main findings of this investigation.

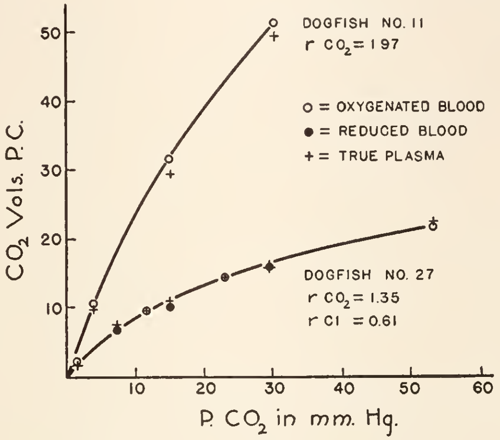

FIG. 1. CO_2 dissociation curves of whole blood and true plasma of dogfish (11) and (27).

RESULTS

The characteristics of the CO_2 dissociation curves of the whole blood will be presented first, then the data relating to the distribution of CO_2 between cells and plasma.

The CO_2 dissociation curves of dogfish blood resemble those of other vertebrates in many respects. When the log of P_{CO_2} is plotted

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against the log of the CO_2 content, a straight line is obtained, except for points at very low pressures of CO_2, i.e. less than 1 mm. Hg. Since analytical errors are magnified at these low values of CO_2 and P_{CO_2}, not much significance can be attached to the discrepancies, although they might be due to carbamino compounds.

The position and slope of the curves vary greatly from fish to fish. The lowest CO_2 content at a CO_2 pressure of 30 mm. was 16 v.p.c.; the highest was 52 v.p.c. Part of this variation was due doubtless to great variation in erythrocyte volume, e.g. from 10 to 25 p.c. It was suspected at first that a seasonal factor was of importance since all of our higher values for CO_2 capacity were obtained early in the summer. A few determinations early in the summer of 1938 showed that this relationship was not invariable.

Oxygenation has no detectable effect on the CO_2 capacity of dogfish blood which is in this respect similar to skate blood (Dill, Edwards and Florkin, 1932). The buffer powers of several samples have been calculated for the range of P_{CO_2} of 5 to 50 mm. Hg by the formula of Van Slyke, B (buffer power) $= \frac{\Delta B}{\Delta \text{pH}}$, ΔB being expressed in millimols/liter. Most of the values varied from 10 to 20 millimols/pH, which agrees well with the values for skate blood. Two specimens (10 and 11), however, showed values of about 90 millimols/pH.

Many samples of venous blood and a few of arterial blood were analysed immediately on removal. The percentage of saturation with O_2 varied in these samples from 25 p.c. to 75 p.c., while the O_2 capacities varied from 2 to 7 v.p.c.; a common value being about 4 p.c. No samples, even of arterial blood, were found to be more saturated than 75 p.c., perhaps because of the relatively high environmental temperature, or of the artificial conditions of aeration of the gills. Nevertheless, one may infer from these data the probable range of CO_2 content and pressure in dogfish blood. The CO_2 content of arterial blood is probably about 6–8 v.p.c. at a tension of perhaps 3 or 4 mm. Hg. The highest value found for CO_2 content of venous blood was 12 v.p.c. at a probable tension of 10 or 12 mm. Hg. Presumably, higher values than this may be attained under conditions of stress. In two specimens, numbers (10) and (11), which had steep dissociation curves, the estimated P_{CO_2} for arterial and venous blood was 2 and 4 mm. Hg respectively. The conditions in these two fish are more like those reported by Dill, Edwards and Florkin for the skate. We can substantiate the comment of these authors that the transport of CO_2 can be accomplished, with relatively small differences in CO_2 tension between venous and arterial blood, in spite of the absence of any effect of O_2 on the CO_2

capacity, because the physiological range of the CO_2 dissociation curve in these forms is on the steepest part of the curve.

Distribution of Carbon Dioxide and Chloride

Table I summarizes the results on distribution of CO_2 and Cl between erythrocytes and plasma. It is apparent that in the majority of specimens the CO_2 content of the erythrocytes per unit of water is definitely higher than that of the plasma in equilibrium with them. The chloride content, on the other hand, is definitely lower in the erythrocytes, which is what one would expect if the conditions of ionic equilibrium were similar to those in mammalian erythrocytes, and determined according to the Donnan Equilibrium. In only one specimen was rCO_2 as low as one. Even in this one, the discrepancy between rCO_2 and rCl is maintained by a low rCl. It should be noted too that the values of rCl are definitely lower than those found at the same pH in mammalian blood. Another point worthy of note is that no tendency was observed for the hematocrit number, rCO_2 or rCl, to vary in any regular fashion with changes in P_{CO_2} ranging from 0 to 60 mm. Hg. For this reason it is justifiable to calculate a mean value for rCO_2 and rCl for the data of one dissociation curve. It should be emphasized, however, that since the hematocrit measurements are rather crude, it cannot be concluded from these data that there is no shift in water or chloride, when CO_2 is added to dogfish blood. This point requires further investigation over wider ranges of P_{CO_2}.

TABLE I

Summary of data on the distribution of CO_2 and Cl between erythrocytes and plasma or serum

Experiment	Number of analyses	Mean rCO_2	Mean rCl	rCO_2/rCl
Dogfish (23)...............	7	1.03	.49	2.11
Dogfish (26)...............	7	1.57	.66	2.46
Dogfish (27)...............	7	1.35	.61	2.23
Dogfish (spiny) (11)........	3	1.97	—	—
Dogfish (spiny) (22)........	3	1.65	—	—
Various venous bloods.......	11	1.46	—	—

Some idea of the regularity of the data may be obtained from Fig. 1 which shows the CO_2 dissociation curves of blood and true plasma of dogfishes (27) and (11). It is seen that the CO_2 contents of whole blood and plasma (and hence of cells and plasma) are practically equal in (27). When allowance is made for the lower water content of the

TABLE II

Complete data of a typical experiment on the heparinized blood of dogfish (23)

	P_{CO_2}	Blood CO_2	Plasma CO_2	Hematocrit	H_2O		Chlorides	
					Plasma	Cells	Plasma	Cells
	mm. Hg	*v.p.c.*	*v.p.c.*		*grams/100 cc.*	*grams/100 cc.*	*m.eq./liter*	*m.eq./liter*
Oxy......	61.3	38.8	40.0	23.4		63.7	253	94
Red......	39.6	34.7	36.3	22.7	89.6	63.7	255	83
Oxy......	31.5	30.6	33.0	21.7	89.4		254	78
Red......	21.0	25.3	27.7	21.8			256	74
Oxy......	16.5	25.2	26.8	23.3			254	90
Red......	12.0	20.3	21.3	22.9	89.7	63.5	256	83
Oxy......	8.2	15.1	16.6	23.1			254	90
				Av. 22.5	89.6	63.6		

cells, it is evident that the concentration of CO_2 per unit of water is higher in the cells than in the plasma. A striking excess of CO_2 in the cells of (11) (a spiny dogfish) is evident. Almost identical results were obtained on (10) (a smooth dogfish). In Table II are presented the complete data of one experiment (dogfish 23). The greatest discrepancies are shown in figures for chloride content of the cells. Inhomogeneity of the samples of packed cells may be responsible for these variations.

DISCUSSION

It is evident that considerably more CO_2 is present in the red cell than can be accounted for by the combined assumptions that the bulk of the CO_2 in the red cell is in the form of free bicarbonate ions, and that the distribution of bicarbonate and chloride ions is according to the Donnan Equilibrium. If it is assumed for the purpose of calculation that the distribution ratio of free bicarbonate ions is equal to that of the chloride ions, then the extra CO_2 in the red cells (Eco_2) may be calculated in approximate fashion as follows:

$$Eco_2 = [CO_2]\ (cells) - [CO_2]\ (plasma) \times rCl.$$

Since rCl and rCO_2 are practically constant over the range studied, Eco_2 increases progressively with Pco_2. This would suggest that the extra CO_2 in the cells was not in the form of carbamate, since carbamino compounds of mammalian hemoglobin are present in maximal concentration at very low pressures of CO_2, and tend to decrease with increasing Pco_2.

Two other possible explanations of the extra CO_2 deserve some consideration. It is possible that some combination of a non-carbamate type may occur between bicarbonate ions and cellular proteins (hemoglobin or others). The evidence for such a compound is discussed in the review by Roughton (1935) under the heading Y-bound CO_2. The likelihood of the existence of such compounds is supported somewhat by the demonstration recently of a specific effect of bicarbonate on the O_2 capacity of hemoglobin by Sidwell et al. (1938).

The other possibility invokes the participation of the nucleus. It has been shown by Chambers and Pollack (1927) that the nuclei of starfish eggs are distinctly more alkaline than the cytoplasm (pH 7.6 as compared with pH 6.8). If this is true in the dogfish erythrocyte one would expect that, at any given pressure of CO_2, the concentration of combined CO_2 would be five or six times greater in the nucleus than in the cytoplasm. If it were further assumed that the nucleus contained little or no chloride, the discrepancy between rCO_2 and rCl could be explained, as well as the low values of rCl relative to mammalian blood. It seems impossible at the present time to decide which, if any, of these views may be correct.

In searching the literature for observations similar to ours, we found the paper of Dill and Edwards (1931) on crocodile blood. They had found an apparent excess of CO_2 in the red cells in both reduced and oxygenated blood. In two respects, however, their findings were different from ours. On reduction of the crocodile blood, the excess CO_2 in the red cell was increased enormously. This suggests that carbamino compounds of CO_2 with hemoglobin may be very prominent in crocodile blood. The other difference, which may or may not be as significant, was that the distribution ratios for chloride were more nearly similar to those in mammalian blood. If the nucleus in these cells were poor in chloride the volume of the nucleus would have to be small compared with the cell volume.

SUMMARY

The CO_2 dissociation curves of dogfish blood (*Mustelus canis*) are in most respects like those of other vertebrates, Log P_{CO_2} plotted against Log $[CO_2]$ gives a straight line.

The CO_2 capacity is not affected by the degree of oxygenation of the hemoglobin.

The oxygen capacities in different specimens varied from 2 to 7 v.p.c.

The tension of CO_2 is estimated to vary, in arterial bloods from 2 to 6 mm. Hg; in venous bloods from 4 to 12 mm. Hg with the CO_2 contents ranging from 6 to 12 v.p.c.

The concentration of CO_2 in the erythrocytes is usually greater than in the surrounding plasma. Reasons are given for supposing the "extra" CO_2 in the red cells *not* to be carbhemoglobin. Two other possible explanations for the extra CO_2 are discussed.

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DIGESTION OF FAT IN AMOEBA PROTEUS

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Greenwood (1886) maintains that if *Amoeba* comes in contact with milk, the fat globules in it are ingested but that they are not digested. She concludes that *Amoeba* does not digest fat. Nirenstein (1905) says there is no evidence which shows that fat is digested by any of the unicellular organisms.

Dawson and Belkin (1928–'29) injected various oils into *Amoeba proteus* and *Amoeba dubia* and then measured the diameter of the globules daily. They found that globules consisting of codliver, olive, cottonseed, linseed, sperm, peanut, oxfoot or oleic oil decreased in size in *Amoeba proteus* and globules of all these, except oxfoot and oleic oils, in *Amoeba dubia,* but that globules of paraffin oil did not decrease in either. They conclude that all these oils except paraffin are digested in *Amoeba proteus* and all except oxfoot, oleic and paraffin in *Amoeba dubia.* It is obvious, however, that the decrease observed in the size of the globules may have been due to oxidation rather than to digestion. Their results consequently do not prove that fat is digested in *Amoeba*.

Mast and Hahnert (1935) studied the digestion of *Chilomonas paramecium* in the food vacuoles in *Amoeba proteus.* They conclude that the fat globules pass from the chilomonads into the surrounding fluid in the vacuole and then out into the cytoplasm but that they probably do not undergo any chemical or physical changes during this process, i.e. that they probably are not digested. They maintain that after the globules of fat pass out of the food vacuoles, they become uniformly distributed through the cytoplasm and finally disappear, presumably owing to oxidation. They consequently obtained no evidence indicating that fat is digested by *Amoeba.*

The following observations were made on the fat in *Colpidium striatum* ingested by *Amoeba proteus.* The colpidia used were cultured in a solution which contained 15 grams tryptone and 2 grams KH_2PO_4 per 1,000 cc. water and an unidentified mold but no bacteria. The mold contained much fatty acid. The colpidia fed on the mold and contained an extraordinarily large amount of neutral fat. The amoebae used were taken from an old declining culture which still contained numerous amoebae. These amoebae contained practically no fat and no food vacuoles except a few in which *Monas punctum* was found. This organism is not digested by *Amoeba proteus,* although it is often ingested in large numbers (Mast and Hahnert, 1935). Two methods were used.

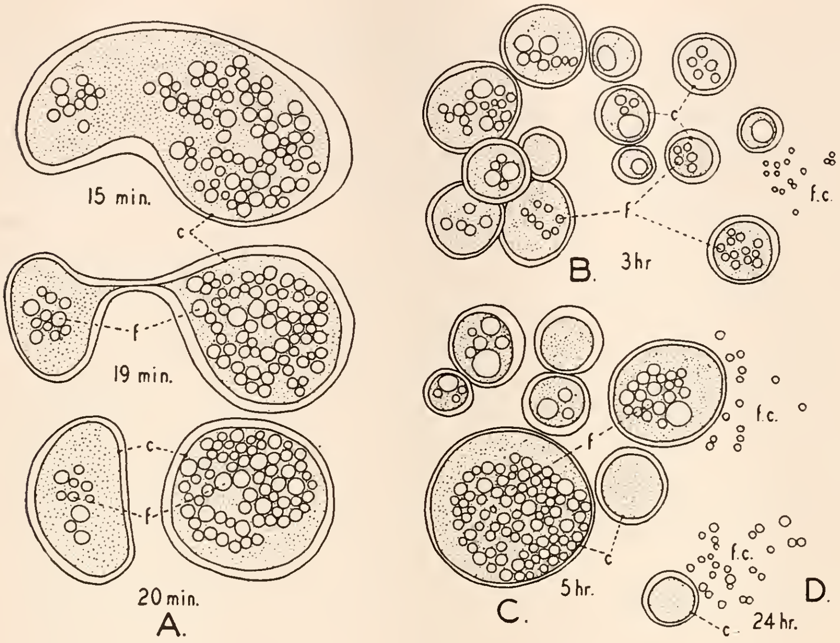

FIG. 1. Camera outlines of food vacuoles in *Amoeba proteus* containing *Colpidium striatum.* *c,* colpidia or fragments of colpidia in food vacuoles; *f,* globules of fat in the colpidia; *fc,* globules of fat in the cytoplasm; 15 min., 19 min., etc., time after ingestion.

A, division of the food vacuole and the colpidium in it; *B,* the food vacuoles and the fat in the cytoplasm of an amoeba three hours after it had ingested a colpidium (Note that the original food vacuole had divided into 13, each containing a fragment of the colpidium, that there were no globules of fat in the solution in the vacuoles around these fragments, that the fat in the cytoplasm of the amoeba was in the form of minute droplets, and that these droplets were much smaller than the globules of fat in the food vacuoles); *C,* the food vacuoles and the globules of fat in an amoeba five hours after it had ingested a colpidium (Note that the globules of fat in the cytoplasm of the amoeba had become larger but that they were still much smaller than those in the food vacuoles and that there was no decrease in size in those but that some vacuoles had none); *D,* the food vacuoles and the fat in the cytoplasm of an amoeba 24 hours after it had ingested a colpidium (Note that the globules of fat in the cytoplasm were well filled with fat and that the globules had increased considerably in size. Some of the amoebae examined had no food vacuoles and some had several but there was no fat in any of them).

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About 250 amoebae were taken from the old culture referred to above. Several of these, taken at random, were stained for fat with Sudan IV. The rest were transferred successively through several separate portions of Hahnert solution;[1] then numerous colpidia were added and left 10 minutes, i.e., until most of the amoebae had ingested one or more colpidia, after which they were again passed through several portions of Hahnert solution so as to remove all the colpidia. An hour later several of the amoebae which contained colpidia were stained for fat with Sudan IV and examined under low and high magnification. This was then repeated from time to time for 24 hours. This entire experiment was repeated three times. There was no essential difference in the results obtained but there was great individual variation in the time required for the processes observed. The following results were obtained in one of the experiments.

1. Six amoebae were taken at random before the colpidia were added, and treated with Sudan IV. No fat was found in two, a few very small globules in one and a considerable number of very minute droplets which probably were fat in each of the rest.

2. Nine were taken 2 hours after having been fed with colpidia and treated with Sudan IV. Preliminary observations showed that soon after a colpidium is ingested the food vacuole usually divides several times, resulting in a number of small vacuoles each of which contains a fragment of the colpidium. Two of the nine amoebae had no food vacuoles but they had a considerable number of minute droplets scattered through the cytoplasm, which probably were fat. Seven had respectively 6, 6, 8, 9, 11, 15 and 21 food vacuoles containing colpidia in various stages of division. A few of these had no fat, but the rest were well filled with large globules. No fat was found in the solution in the food vacuoles, around the fragments of colpidia.[2] Three of the amoebae had a few small fat globules in the cytoplasm and the rest had

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[1] Hahnert solution is composed of KCl .004 gram, $CaCl_2$ .004 gram, $CaH_4(PO_4)_2 \cdot 2H_2O$ .002 gram, $Mg_3(PO_4)_2 \cdot 4H_2O$ .002 gram, $Ca_3(PO_4)_2$ .002 gram, and H_2O 1,000 cc.

[2] Mast and Hahnert (1935) maintain that the globules of fat in chilomonads in food vacuoles in amoebae pass out into the surrounding solution in the vacuoles. In observation on colpidia in amoebae which were under cover-glasses with but little space between them and the slides I repeatedly saw globules of fat come out of the colpidia especially when pressure (even if only slight) was applied to the cover-glass, but I never saw any indications of it in preparations in which the cover-glasses were supported so as to leave abundance of space under them. It is probable, therefore, that the movement of globules of fat out of the chilomonads observed by Mast and Hahnert was due to external pressure.

minute droplets scattered through the cytoplasm, like those which had no food vacuoles.

3. Nine were taken 3.5 hours after having been fed. Two of these had no food vacuoles and probably no fat in the cytoplasm. The rest had respectively 4, 9, 12, 13, 15, 16 and 18 food vacuoles. Two of these had very minute droplets probably fat. Each of the rest had a considerable number of small globules of fat scattered through the cytoplasm but these globules were much smaller than those in the fragments of colpidia in the food vacuoles.

4. Nine amoebae were taken 5.5 hours after having been fed. All of these had food vacuoles, the number varying from 3 to 17. One of the amoebae had minute droplets in the cytoplasm, probably fat. Eight had small fat globules scattered through the cytoplasm.

5. Six amoebae were taken 7 hours after having been fed. All had food vacuoles, a total of 50. Eighteen of them had fragments of colpidia which contained large globules of fat. The rest (32) had no fat. Four of the amoebae had numerous small fat globules scattered through the cytoplasm. One had a moderate number and one only a few. None had minute droplets. All the globules of fat in the cytoplasm were much smaller than those in the food vacuoles.

6. Eight amoebae were taken 24 hours after having been fed. Only one of the 8 had any food vacuoles and that one had only four and there was no fat in any of them. One had no fat in the cytoplasm. All the rest had numerous globules scattered through the cytoplasm (Fig. 1).

The results presented show that soon after a colpidium is ingested the food vacuole and its content divides and that this is repeated until there are several, each containing a portion of the colpidium. They show that the globules of fat in the fragments of colpidia do not pass out into the solution around them in the vacuoles but that they gradually decrease in number until all have disappeared and that as they decrease in number, globules of fat appear in the cytoplasm of the amoeba and increase in size and number but that they are continuously much smaller than those in the food vacuoles.

This proves that the fat does not leave the food vacuoles in the form of globules and that it is digested, passes out into the cytoplasm in solution and is there resynthesized.

II

About 200 amoebae were taken from the old culture used in the preceding experiments and put into Hahnert solution; then numerous colpidia were added. After 2, 4, 9 and 24 hours respectively, some of the amoebae which had ingested colpidia were taken out and put into Hahnert solution and stained with Nile blue sulfate for fat and fatty acid. Some colpidia which had not been ingested, some amoebae from a young culture and some emulsified oleic acid were also stained with Nile blue sulfate.

The oleic acid became densely purplish blue. The fat in the colpidia and that in the cytoplasm of the amoebae became pink, indicating that it was neutral. There was no bluish tinge in any of it, i.e. there was no indication of fatty acid. The fat in the colpidia which had recently been ingested was just as pink as that in the colpidia which had not been ingested and that in the cytoplasm of the amoebae. That in the colpidia which had been in the food vacuoles 2 or more hours was lavender or purple. The shade varied considerably in different vacuoles but it was *clearly* bluish in all. (The color of the fat in these vacuoles was repeatedly compared with that of the fat in the cytoplasm of the amoebae and that in colpidia which had recently been ingested, and it could definitely be seen that there was a marked difference in color, the former being definitely bluish.) The fat in the oldest (smallest) food vacuoles was, however, not appreciably bluer than that in younger vacuoles. It was not distinctly blue in any, not blue like the blue obtained in emulsions of oleic acid.

These facts indicate that the fat in *Colpidium* is changed to fatty acid and glycerine, that these substances leave the vacuoles soon after they are formed, and that in the cytoplasm of the amoebae they unite and form neutral fat. There is no evidence indicating that fatty acid accumulates to any considerable extent in the vacuoles. Small amounts of fat in old vacuoles are no bluer than large amounts in younger vacuoles.

SUMMARY

1. If a colpidium which contains numerous globules of fat is ingested by an amoeba which contains no fat, the food vacuole and its content soon divide several times forming a number of vacuoles, each containing a fragment of the colpidium.

2. The globules of fat in these fragments gradually disappear and as they disappear, small droplets of fat appear in the cytoplasm of the amoeba, which gradually increase in size and number until in 24 hours all the fat in the fragments has disappeared and the cytoplasm of the amoeba is well filled with globules of it.

3. Treatment with Nile blue sulfate shows that the fat in the intact colpidia and that in the cytoplasm of the amoebae is neutral, but that

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the fragments of colpidia in the food vacuoles contain fatty acid as well as neutral fat.

4. The fact that the globules of fat in the fragments of colpidia in the food vacuoles are much smaller than those in the cytoplasm of the amoebae when first observed, shows that they do not pass out into the cytoplasm and it indicates that the fat is digested in the fragments and passes out in solution. The fact that fatty acid is formed in the fragments indicates that the fat in them is split into fatty acid and glycerine which pass out into the cytoplasm and unite there to form neutral fat.

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THE OXYGEN-CONSUMPTION OF COLPIDIUM CAMPYLUM

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Introduction

Interest in the respiratory exchange of Protozoa dates from the work of Vernon (1895) on the radiolarian, *Collozöum inerme*. In the forty-three years that have elapsed since the publication of Vernon's paper, observations have been made on the gas-metabolism of species belonging to three of the four major groups of unicellular animals. During this period, however, the development of protozoölogical technique has lagged behind the very rapid improvement in physiological and biochemical procedure, so that much of the data which have accumulated are difficult or impossible to evaluate.

Beginning with Lund's (1918*b*) work on *Paramecium caudatum*, oxygen-consumption of ciliate protozoa has repeatedly been found to be relatively unaffected by cyanide, suggesting that the mechanism by which these forms use molecular oxygen differs fundamentally from the usual one found in aerobic cells. The nature of the causes underlying this difference should prove to be of considerable general interest. It seemed worthwhile, therefore, to begin a careful study of the intracellular oxidative mechanism in a species of ciliate. The holotrichous form *Colpidium campylum* was chosen for the purposes of this investigation. In order critically to study the respiratory mechanism, it was necessary to have precise measurements of the normal rate of oxygen-consumption. The existing data provide no such sound basis for further experimental work with this species. Accordingly, the rate of oxygen-consumption of *C. campylum* has been determined manometrically under carefully controlled and, therefore, reproducible conditions.

The current paper is a contribution to the growing literature in a relatively new field of investigation dealing quantitatively with the biochemical properties of pure suspensions of protozoa. Invaluable data have resulted from the application of such methods of study in the field of bacteriology. Their extension to the Protozoa, although highly desirable in view of the biological and medical importance of this phylum, was formerly rendered impracticable by the complexity of the media required for cultivation of most protozoan forms. With the development of suitable isolation methods and of artificial media capable of supporting growth, a limited number of species are now available in bacteriologically sterile, non-particulate cultures. Such cultures offer material for biochemical investigation which is more homogeneous and free from specialization of cellular function than metazoan tissue slices.

This research was carried out under the supervision of Professor Gary N. Calkins of the Zoölogy Department of Columbia University. The author wishes to express his appreciation of the interest which Professor Calkins has maintained in the progress of the work, and to acknowledge his indebtedness to him for his aid in preparing the manuscript.

Material and Methods

A bacteria-free culture of *Colpidium campylum* (Stokes) Breslau was obtained from the Lenox Hill Hospital through the courtesy of S. M. Nagy. This strain was originally isolated by Hetherington and identified by Lwoff. Cultures of the ciliates were grown in 20 × 150 mm. cotton-plugged Pyrex tubes containing 15 cc. of sterile medium. Transfers were made with sterile volumetric pipettes of 1 ml. capacity, observing the usual precautions in order to prevent bacterial contamination. The medium employed for cultivation of the organisms consisted in a 2 per cent solution of tryptone (Difco) containing 0.1 per cent yeast-extract (Difco), made up in the following salt solution:

KH_2PO_4 (Sørensen)	0.5 gram
NaCl	0.4 “
$MgSO_4.8H_2O$	0.1 “
$Ca(NO_3)_2.4H_2O$	0.1 “
Distilled water	1,000 cc.

The reaction of the medium after autoclaving was pH 6.6 ± 0.02. The pH-measurements were made with a quinhydrone electrode connected through a saturated KCl-agar bridge to a saturated KCl-calomel half-cell.

The experimental suspensions were made up with material from cultures incubated 48 hours in a thermostat-controlled room at 23° C. The supernatant layer of actively swimming ciliates was poured off the top of a culture into a clean centrifuge tube, being careful not to stir up the precipitate of dead organisms at the bottom. Material from several cultures was concentrated by centrifuging and transferring to a single clean test-tube. Suspensions prepared in this manner cannot be described as absolutely bacteria-free, since the cultures from which they are made up undoubtedly become contaminated as soon as they are exposed directly to the air. For practical purposes, however, such suspensions may be regarded as bacteria-free, since the number of bacteria present even after several hours is entirely negligible in comparison with the magnitude of the protozoan population.

In the present investigation the rate of oxygen-consumption by *Colpidium campylum* is stated in terms of the number of cubic millimeters of gas, reduced to 0° C. and 760 mm. pressure, taken up per million cells. When results are expressed in this manner, their accuracy is largely determined by the precision with which the number of cells in the experimental suspensions is estimated. The following procedure has been found satisfactory. By means of a calibrated pipette, samples were withdrawn from the concentrated suspension alternately to be used in performing the measurements and in estimating the number of cells. The pipette used in taking samples delivered 0.995 cc. at room temperature. Before taking each sample, the tube containing the concentrated suspension was rolled vigorously between the palms of the hands or flipped carefully to insure uniform distribution of the contents. Before any samples were taken the pipette was first wet by drawing up some of the suspension. This procedure helps to correct for the tendency of the cells to stick to the sides of the pipette. As a rule, two samples were transferred into the main chamber of each manometer-vessel. The samples to be used in determining the concentration of the suspension were placed in clean test-tubes containing 5 drops of Bouin's alcoholic fixative. Each tube received a single 0.995 cc. sample; 9 cc. of distilled water were added from a calibrated burette, and the tubes were plugged with cotton and set aside in the refrigerator. When counts were to be made, the tubes were removed and shaken vigorously. Single samples from each were placed in separate clean test-tubes, employing the same pipette which was used in taking the original samples from the experimental suspensions. The contents of each of these tubes were diluted with 9 cc. of distilled water from the calibrated burette, and counts were made on 0.995 cc. samples withdrawn from the resulting suspensions after thorough shaking. A Sedgwick-Rafter counting chamber of the circular type was used. In order to facilitate counting, the bottom surface of the chamber was ruled off into 5 mm. squares with a diamond-point pencil. The number of ciliates in the entire contents of the chamber was counted under a binocular dissecting microscope, using the 2.3 × objective and 9 × oculars. The results of the several counts were averaged and multiplied by 100 to obtain the average number of cells in each sample taken from the original concentrated suspension. This figure, multiplied by the proper small integer representing the number of samples placed in each manometer vessel and expressed as a decimal part of one million, was used in calculating the rate of oxygen-consumption. The difference between the counts obtained with individual samples and the average for all samples taken from the same original suspension was taken as an index of the probable error of counting. From the differences obtained in this way in each experiment the average percentage difference between counts performed on samples from the same suspension was calculated. This constant is a mixed one including both the sampling error and the error of counting.

Oxygen consumption was measured directly, using Warburg manometers fitted with rectangular vessels similar to those employed by Warburg, Kubowitz and Christian (1931), but with an inset chamber for potassium hydroxide. The theory of the method has been adequately summarized and appraised by Dixon (1934). Hence, only those details will be discussed here which are of particular importance in connection with this investigation. In the majority of the experiments four or five manometers were used, one of these in each case serving as a thermo-barometric control. The average capacity of the manometer vessels was 8.812 cc. to the level of the manometric fluid (Brodie's solution colored with Sudan III), and the average volume of the gas space was 6.689 cc. The manometer constants were calculated by substituting in the formula derived by Warburg. The total volume of oxygen consumed was obtained from the algebraic difference between the observed change on the scale of the experimental manometer and that on the scale of the thermobarometer by multiplying by the proper manometer constant. The rate of oxygen-consumption was calculated as the quotient obtained by dividing the total volume of oxygen consumed in unit time (1 hour) by the total number of cells in the suspension expressed as a decimal part of one million. In order to insure efficient absorption of the carbon-dioxide evolved during respiration, small rolls of No. 40 Whatman (starch-free) filter paper were placed in the inset chambers of the vessels and soaked with 20 per cent carbonate-free KOH. The volume of alkali added was 0.133 cc. in every case. The temperature was controlled by immersing the vessels in a water bath at 19.8° ± 0.02° C. The manometers were attached to a shaking device and shaken constantly through 5 cm. of arc, except during the time necessary for taking readings.

Experimental Results

Dixon and Elliott (1930) have shown that in performing manometric measurements, if the results are to represent the true gaseous exchange of the tissue or cell-suspension, certain purely mechanical factors must be taken into consideration. Under certain circumstances the capacity of the manometer vessels and their shape, together with the rate at which the manometers are shaken, may determine the accuracy of the measurements. The manometers should be shaken rapidly enough to render oxygen-consumption of the contained tissue or cell-suspension independent of the diffusion equilibrium between the gas space and the fluid bathing the tissue or cells.

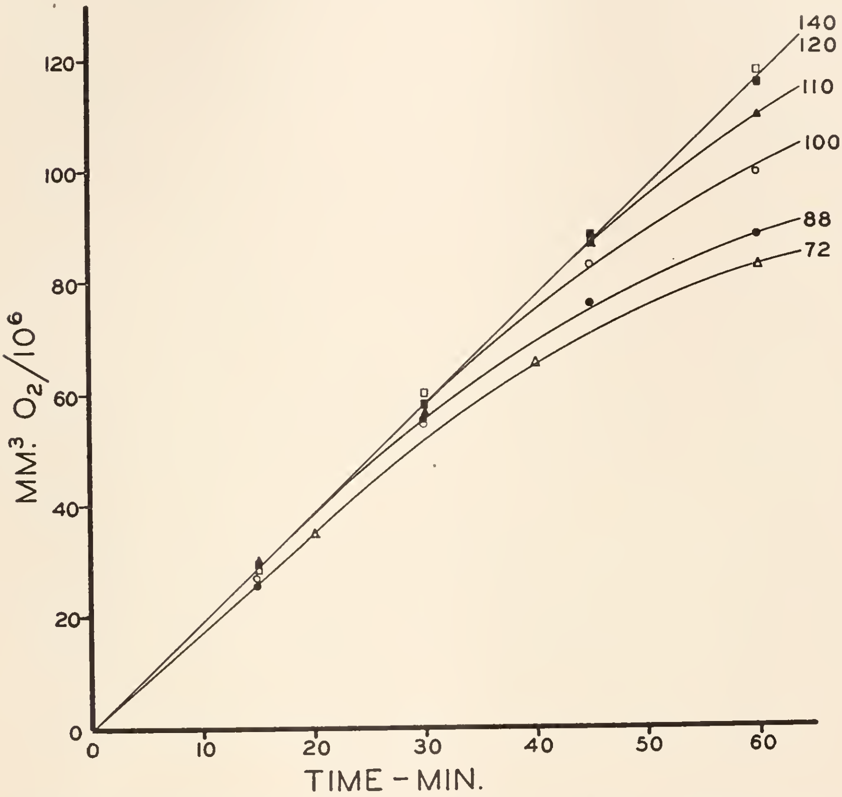

Fig. 1. Oxygen-consumption by suspensions of *C. campylum* at different rates of shaking. Ordinate—cu. mm. of O_2 per million protozoa; abscissa—time in minutes. The figures at the right, opposite the curves, refer to the rate of shaking expressed as the number of complete cycles per minute.

At the beginning of the present investigation, therefore, a series of experiments was carried out with the object of finding a suitable rate of shaking for suspensions of the proper concentration to insure obtaining significant readings. The results are shown in Fig. 1. Each point represents the average of the data obtained with three different manometers except for the experiment in which the rate of shaking was

140 cycles per minute, where an unfortunate accident eliminated two of the manometers. The average number of cells in the suspensions was about 250,000. From the data obtained it is clear that with suspensions of the order of density of a quarter of a million cells the rate of oxygen-uptake is dependent on the rate of shaking until a frequency of about 120 cycles per minute is attained. Accordingly, in all subsequent experiments the manometers were shaken at a rate of 120 cycles per minute.

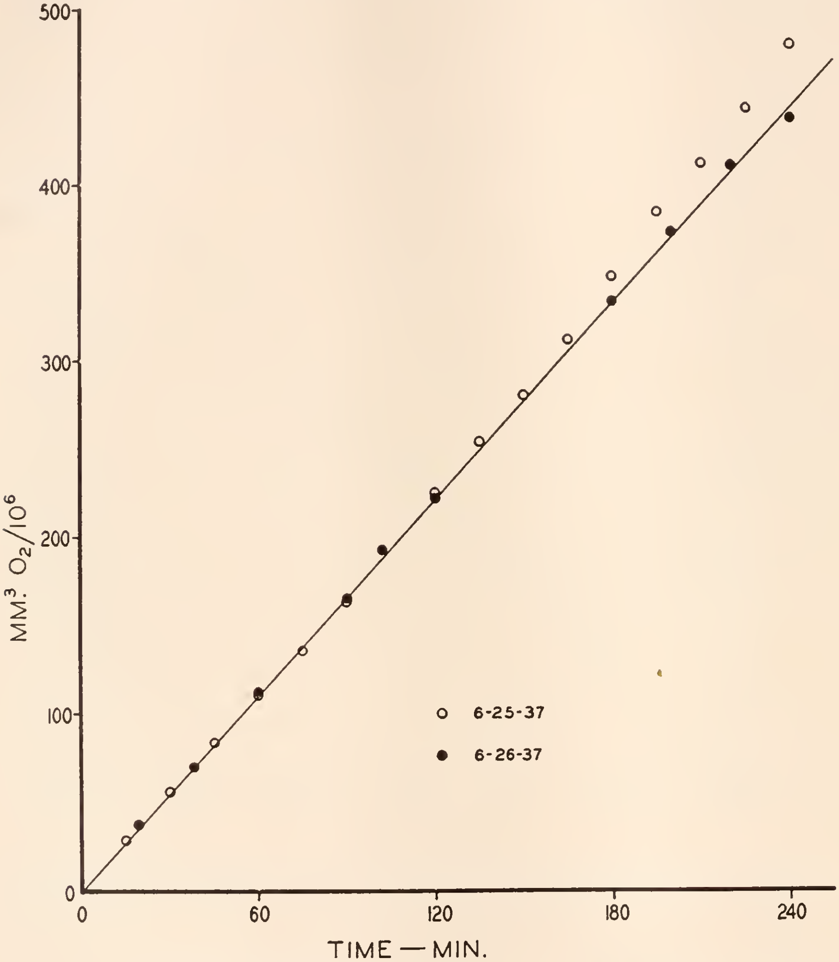

Fig. 2. Oxygen-consumption by suspensions of *C. campylum*. Data of single experiments performed with the same manometer. Ordinate—cu. mm. of O_2 per million protozoa; abscissa—time in minutes. Explanation in text.

In Fig. 2 are shown the results of two experiments, performed on successive days with the same manometer, in which the oxygen-consumption of suspensions of *Colpidium campylum* was followed during a period of over four hours. The duration of individual runs was one hour. The concentrations of the experimental suspensions were approximately the same, about 230,000 cells in each case. The open circles in Fig. 2 represent the data obtained June 25, 1937. In this experiment tap-water was used in the vessel of the manometer which served as a thermobarometric control. A gradual increase in the rate of oxygen-consumption is indicated after the second hour. Since the increase was very gradual, it seemed likely that it might be only an apparent one brought about through the cumulative effects of autoxidizable substances and bacterial contamination in the suspension, or by differences between the solubilities of the respiratory gases in tap-water and in culture fluid. All of these effects could readily be compensated by using fresh culture medium in the vessel of the thermobarometer. This was done in the experiment performed the following day, the data of which are represented by the filled circles in Fig. 2. It is evident that when the proper precautions are taken in setting up an experiment the observed rate of oxygen-consumption by suspensions of *C. campylum* remains constant over a period of at least four hours. The average rate computed from the data of June 26 is 111.0 mm.^{3} O_2/hr./million.

In order to obtain critical measurements of the rate of oxygen-consumption, a series of experiments was performed in which three or four samples were tested in each run. A summary of these experiments is given in Table I. By averaging the results of the 36 measurements included in the table, a value of 112.5 mm.^{3} O_2/hr./10^6 is obtained for the rate of oxygen-consumption by suspensions of *Colpidium campylum* in the medium employed for their cultivation. Comparison of this figure with that of 111.0 mm.^{3} O_2/hr./10^6 given in the preceding paragraph shows that individual runs yield data of good precision.

The data of Table I provide an index of the accuracy with which samples of like density can be taken from the same original concentrated suspension, using the technique described in this paper. The standard deviation between the oxygen-quotients obtained for different samples in the same run was 0.0194. Since the combined error of counting and sampling is of the order of about 4 per cent, it is evident that the sampling error falls well within the limits of the general method of relating the volume of oxygen consumed to the number of respiring cells. The data show that no significant difference in results is obtained with different manometers provided the latter are accurately calibrated. While the average result obtained with manometers Nos. 203 and 205 was higher than with the other two manometers, the difference does not appear consistently and represents only about 1.2 per cent of the average hourly uptake, whereas for the 36 measurements included in the table the standard deviation of the average $Q_{\bar{O}_2}$-value is 0.0363. In two of the experiments included in Table I it will be noted that suspensions of apparently greater density were used in manometers Nos. 203 and 205 than in the other two manometers in the same runs. In preparing these experiments, different volumes of the original concentrated suspension were measured into the vessels of the respective manometers. The total volume of the suspension, and, hence, the surface-volume relationship, were thus varied without altering the actual density of the suspensions. This was done in order further to check

Table I

Summary of the data of eleven experiments in which three or more samples were tested simultaneously. Duration of each run = 1 hour. Temperature = 19.8° ± 0.02° C. Gas-mixture — air. Rate of shaking = 120 cycles/minute.

Date	Hour	No. of cells $\times 10^{-6}$	$Q_{\bar{O}_2}$ — mm.^{3} $\bar{O}_2$/hr./10^6				
			Manometer number				Average
			200	202	203	205	
6–2	1	0.226	109.4	106.2	111.1		108.9
	2		110.3	103.0	111.9		108.4
6–5	1	0.139		117.5	116.0	113.8	115.7
	2			118.8	118.4	119.7	119.0
6–21	1	0.238	116.0		113.9	117.4	115.7
7–1	1	0.247	111.2		110.2	112.0	111.1
	2		109.2		110.4	106.4	108.7
7–7	1	0.321	111.3		113.4	121.3	115.3
7–14	1	0.234	106.8	110.3			108.5
		0.466			108.9	109.0	109.0
7–21	1	0.251	116.7	113.0			114.9
		0.376			113.9	109.3	111.6
	2	0.251	114.9	114.1			114.5
		0.376			114.4	110.1	112.3
Averages		0.284	111.8	111.8	113.0	113.2	*112.5*

whether the rate of shaking (120 cycles per minute) was adequate to insure uniform results when larger populations were to be tested. The data do not provide any evidence to the contrary.

Discussion

Comparison of the data presented above with those of earlier workers is difficult because of the variety of conditions, often imperfectly known, under which previous measurements have been carried out. Table II is a summary of the results obtained by other investigators where comparable procedures have been employed and where the data could be expressed in a standard way as the volume of oxygen in cubic millimeters consumed per million cells per hour.

Investigations have ranged in exactness from observations on the ability to survive under conditions of anaerobiosis (Pütter, 1905) to determination of the gaseous exchange of single organisms in a capillary micro-respirometer (Kalmus, 1927, 1928; Howland and Bernstein, 1931). Efforts toward quantitative measurements began, although they attained no accuracy, with the use of absorption apparatus (Barratt, 1905) and various modifications of the Winkler technique (Wachendorff, 1912; Lund, 1918). Von Fenyvessy and Reiner (1924), Soule (1925) and Amberson (1928) probably obtained more accurate results by carefully analyzing samples of gas from sealed cultures, using the Barcroft-Haldane-Henderson apparatus. Unfortunately, none of these investigators attempted to express his findings in quantitative terms. Beginning with the experiments of Błedowski and Zweibaum (1915) the majority of studies have involved the use of manometric methods.

The earliest measurements were made on material from mixed cultures in media of uncertain composition. The disadvantages of such cultures have been discussed elsewhere (Jahn, 1931; Phelps, 1931, 1935; Raffel, 1932) in connection with growth studies. In attempting to measure respiration of protozoa in mixed suspensions, particular difficulties are encountered, since the metabolism of the bacteria or other foreign microörganisms may alter the results directly to an indeterminate extent. The importance of this factor is brought out by comparison of the following data. Cook and Haldane (1931) have shown that at 16° C. *B. coli communis* consume 17.5 $\text{mm.}^3\ \overline{O}_2$/hr./mg. bacterial nitrogen in the absence of any oxidizable substrate. Since these bacteria contain about 8.3 per cent of their dry weight of nitrogen, the average basal oxygen-uptake is 1.5 $\text{mm.}^3\ \overline{O}_2$/hr./mg. dry weight. ($Q_{\overline{O}_2}$ is obtained by multiplying by 0.083—not by dividing, as stated by Cook and Haldane—and is of the same order of magnitude as for yeast or mammalian tissues.) In the presence of an oxidizable substrate, *e.g.*, lactate, $Q_{\overline{O}_2}$ becomes 12 mm.^{3} $\overline{O}_2$/hr./mg. dry weight. When *B.*

Table II

Summary of the Results of other Investigators

Species	Investigator	Temp.	$Q_{\overline{O}_2}$	Remarks
		°C.		
Actinosphaerium eichhorni	Howland, 1931	20.0?	1130.0	Bacteria present
Chilomonas paramecium	Mast, Pace and Mast, 1936	25.0	16.4	Bacteria-free suspensions
Colpidium campylum	Pitts, 1932	24.0	151.5	Bacteria for food
C. colpoda	Wachendorff, 1912	17.0	191.0	1 day culture
			151.0	10 day culture
			59.0	30 day culture
		7.0	33.0	
C. colpoda	Peters, 1929	25.0?	200.0	Bacteria-free suspensions?
Colpoda sp.	Adolph, 1929	19.8	575.0	Clone "B"
			537.5	Clone "C"
			1185.0	Clone "E"
Paramecium caudatum	Lund, 1918	25.0	140.0	Fed
			40.0	Starving
	Zweibaum, 1922	23.0	737.0	Before conjugation
			3481.0	During conjugation
			2142.0	After conjugation
	Necheles, 1924		3850.0	
	Kalmus, 1927	21.0	5200.0	Single cells
	1928	17.0	4600.0	Single cells
		13.0	3300.0	Single cells
Paramecium caudatum	Howland, 1931	20.0	490.0	Single cells
P. multimicronucleatum	Mast, Pace and Mast, 1936	25.0	1021.0	Bacteria present
Spirostomum ambiguum	Specht, 1935	25.0	2590.0	

coli oxidizes formate, the rate of $\overline{O}_2$-uptake is about 40 mm.^{3} $\overline{O}_2$/hr./mg. dry weight of bacterial suspension. The holotrichous ciliate *Glaucoma pyriformis* consumes on an average about 35 mm.^{3} $\overline{O}_2$/hr./mg. dry weight at 22° C. in 2 per cent peptone-water (M. Lwoff, 1934). In order to overcome the disadvantages of mixed suspensions, recourse has been made in the past to diminishing the number of bacteria, etc., by washing (Wachendorff, 1912; Necheles, 1924; Adolph, 1929; Emerson, 1930; Specht, 1935), or to balancing the respiration of the mixed population against that of a suspension of the bacteria alone (Zweibaum, 1921; Grobicka and Wasielewska, 1925; Gerard and Hyman, 1931). Bacteria-free cultures of protozoa were first employed in measurements of respiration by Soule (1925).

In most previous investigations no atttempt has been made to control the reaction of the medium in which the organisms were suspended, and the relation between pH and the rate of oxygen-consumption is not known. In only a few instances has the age of the population been taken into consideration, although the importance of this factor was early recognized (Wachendorff, 1912). Phelps (1935, 1936) has shown that growth of *Glaucoma pyriformis* in bacteria-free cultures resembles that of yeast, Protophyta, and bacteria. There is a logarithmic phase during which the population increases at a constant rate, a phase of negative acceleration when the rate of increase in the population decreases although the total number of cells continues to increase, and a stationary state in which the death rate just balances the rate of increase. The suspensions of *Colpidium campylum* employed in the present investigation were made up with material from cultures in the stationary growth phase. It is reasonable to suppose that significantly different results might have been secured with material from cultures in the logarithmic phase or that of negative acceleration of growth.

There are no reliable data correlating the oxygen-consumption of protozoa with temperature, although measurements have been made at temperatures ranging from 6.5° to 28° C. Wachendorff's (1912) results indicate that the rate of respiration of *Colpidium colpoda* is quadrupled by an increase of 10° C. in temperature. From the results of experiments with single *Paramecium caudatum* at 13°, 17°, and 22° C., Kalmus (1928) concluded: "Wie die bisher untersuchten messbaren Lebensvorgänge des Objekts, folgt auch die Atmung in der Umgebung des Temperatur-optimums der RGT-Regel."

Wachendorff (1912) and Lund (1918) employed the Winkler technique for determining oxygen-consumption. In spite of the fact that this method is hardly adapted for the precise estimation of minute changes in oxygen-tension, the measurements were made with suspensions of only a few thousand cells. In order to obtain significant readings it was necessary to prolong the experiments over a period of 6–24 hours, during which interval the increase in bacterial flora must have been sufficient to influence the results markedly even if the protozoa themselves failed to divide. The work of Necheles (1924), Adolph (1929) and Specht (1935) is subject to criticism of the same sort. These investigators have used manometric methods in measuring the oxygen-consumption of mixed suspensions containing between 900 and 12,000 cells. With suspensions of such concentrations measurable changes are produced only after relatively long periods of time. Consider the data obtained in the present investigation. At the rate of 112.5 mm.^{3} O_2/hr./million, 5,000 cells would use 0.5625 mm.^{3} of oxygen in an hour. Since the average value of the gas constant for the manometers employed in this research was 0.661, the average change produced on the manometer scale by 5,000 cells in one hour would be about 0.836 mm. With the aid of a hand-lens, readings can be made to the nearest 0.1 mm.; hence, the error introduced in such measurements in taking readings alone would be of the order of magnitude of 12.0 per cent.

The first measurements of the oxygen-consumption of Protozoa made under satisfactory conditions are those of A. Lwoff (1933). These experiments were done at 28° C. with suspensions of *Strigomonas fasciculata, S. oncopeltis* and *Leptomonas ctenocephali* from 96-hour bacteria-free cultures in 2 per cent peptone solution at pH 7.0. The data obtained by M. Lwoff (1934) for *Glaucoma pyriformis* are the first dealing with the oxygen-consumption of a ciliate under adequately controlled conditions. Unfortunately, the Lwoffs have expressed their findings in terms of the volumes of oxygen consumed per milligram dry weight of the suspension, so that it is impossible to compare their results with those either of the current research, or of other investigators.

Summary

A procedure has been described for obtaining accurate measurements of the rate of oxygen-consumption of Protozoa. This procedure involves the use of Warburg manometers and concentrated suspensions of organisms made up from bacteria-free cultures. The rate of oxygen-uptake by the holotrichous ciliate *Colpidium campylum* has been determined. The average of 36 measurements gave a value of 112.5 mm.^{3} $\bar{O}_2$/hr./million for the rate at 19.8° C. in the medium in which the infusoria had been growing for 48 hours. The rate remained constant over a period of more than four hours. The standard deviation of the measurements was 0.0363.

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THE INFLUENCE OF CENTRIFUGAL FORCE ON THE BILATERAL DETERMINATION OF THE SPIRALLY-CLEAVING EGG OF URECHIS

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INTRODUCTION

There is evidence from the experiments of several workers, particularly Runnström (1926), Lindahl (1932), and Pease (1938), that the location of the plane of bilateral symmetry of various echinoderm eggs can be influenced by centrifugal force. It is of considerable interest, then, to determine whether this can be done with a spirally cleaving egg. The observations on cell lineage have led investigators to believe that, in general, in such eggs the position of the first cleavage plane determines the plane of bilateral symmetry. Further, it has been found by Just (1912), Morgan and Tyler (1930), Tyler (1931), and others that in such eggs the sperm entrance point is related to the first cleavage. However, Hörstadius (1937) has shown by vital staining that in the *Cerebratulus* egg the first cleavage may bear any relation to the bilateral axis. Furthermore, he concludes from the development of partial embryos that there is no evidence of bilateral determination at the two- or four-cell stage such as was indicated in the echinoderm egg by the experiments of Hörstadius and Wolsky (1936).

Eggs of the spirally cleaving type have been centrifuged by various workers including Boveri (1910), Conklin (1910, 1916), Hogue (1910), Lillie, F. (1909*a* and *b*), Morgan (1910), von Parseval (1922), Schleip (1914), Tyler (1930) and Wilson (1929 and 1930). None, however, have reported any influence of the stratification on the position of the median plane of the embryo. In the early experiments the centrifugal forces employed were relatively low. Also counts of the various types of embryos resulting were not made, it having been considered at best sufficient to demonstrate that embryos might be obtained with the different layers in any region. Much of the work was entirely unconcerned with the possibility that centrifuging could influence the bilateral determination.

I have therefore examined these questions in the *Urechis caupo* (Fisher and MacGinitie) egg using the high centrifugal forces possible with an air turbine ultracentrifuge. The results show a definite relation between the axis of stratification and the plane of bilateral symmetry in the resulting embryo. It is concluded that the stratification of material substances induces the ventral side near the centripetal end.

My thanks are particularly due to Dr. Albert Tyler of the California Institute of Technology for his aid and suggestions during the course of the work and for his critical reading of the manuscript of this paper.

Material and Methods

The eggs of the echiuroid worm, *Urechis caupo* (Fisher and MacGinitie), were used in these experiments. A brief description of the cleavage and trochophore stages has been given by Tyler (1931). The development is very similar to that of *Thalassema mellita* (Conn.) of which Torrey (1903) has carefully worked out the cell lineage.

The eggs were subjected to varying high centrifugal forces in an air turbine ultracentrifuge for short periods of time. They were suspended in the centrifuge tubes in about six parts of isotonic sucrose and four parts of sea water. This mixture was found to very nearly equal the specific gravity of the eggs. In calculating the forces applied, the speed of the rotor was known and the radius measured to the approximate position of the eggs in the centrifuge tubes. The forces are listed to the nearest thousand gravities and are within 10 per cent of the calculated figure. The eggs were centrifuged both before and after fertilization. Except where noted in the results, the viability of the centrifuged eggs seemed to approximate that of the controls and there was no indication of a differential mortality. The hardest centrifuging did not activate the eggs.

To study the eggs and embryos they were fixed in strong Flemming's solution, in general for about twelve hours, and then were washed for another twelve hours in water. Temporary glycerine preparations were then made. The oil during centrifuging goes to the centripetal end and occupies almost one-quarter of the periphery of the egg. It is stained jet black by this treatment and retains this color a number of hours when the eggs or embryos are cleared directly in glycerine. The black stain of the oil is by no means permanent in the glycerine and the embryos must be examined within a few hours after clearing them. The diffusion of the oil is so slow in the egg that a very good boundary is maintained until the trochophore stage. This in no way seems to effect the viability, and this condensed oil mass was found in otherwise normal trochophores in all regions of the embryo. It does not block cleavage and the cells containing great excesses of oil do not seem by superficial inspection to be abnormally large.

The centrifuging makes four primary layers in the unfertilized egg. Most centripetal is the large "oil cap," occupying about a quarter of the circumference. There follows a broad layer making up more than half of the egg by volume and about a half of the circumference. This contains the germinal vesicle which comes to lie just below the oil cap. The layer is fairly clear and can be recognized as a double, if not sometimes a triple layer. The centrifugal quarter of the circumference is occupied by the third and fourth layers. The third contains the bulk of the yolk and occupies most of the space. The fourth layer is made up of coarse pigment granules.

In studying the trochophores only those were considered that were normal or nearly so. Aberrant types were found to be difficult or impossible to orient. The aberrations found among the trochophores of the centrifuged eggs resembled those found in the control larvae and, in general, except when specifically indicated in the results, were no more common in the experimental larvae than in the controls. There is no indication that in eggs centrifuged before fertilization and in some experiments following fertilization that there is any differential mortality or aberration.

In those counts that were made to determine whether the centrifuging influenced the bilateral axis, only those embryos were counted that had the centripetal oil in the prototroch or trochophore cilia band. Similarly, to determine whether there was any influence on the polar axis, counts were made of trochophores that had the oil in the region anterior to the prototroch derived normally from the animal hemisphere and those with the oil in the post-trochal and gut regions normally derived from the vegetative hemisphere. The first hundred embryos seen in any experiment were grouped into types and the number found of any type is therefore the percentage for that experiment based on a random count. Any exceptions are specifically explained in the results.

RESULTS CONCERNING THE BILATERAL DETERMINATION

Different forces were applied for different lengths of time in ten experiments on the unfertilized eggs. Directly after centrifuging the eggs were fertilized. The data obtained are summarized in Table I. The categories in which the trochophores were grouped are, of necessity, somewhat arbitrary. Only trochophores with the oil present in the prototroch are recorded. In the first group it was found in the nearly median plane on the ventral side. In the second group it was ventral oblique, in the third nearly lateral, and in the fourth group dorsal or dorsal oblique.

As will be seen in Table I, of a thousand embryos counted at random and grouped into these categories, 41.1 per cent had the centripetal oil in the ventral median position, 33.2 per cent had the oil in the ventral oblique position, 21.5 per cent had it in a lateral position, and only 4.2 per cent had the oil dorsal or dorsal oblique. It is quite clear from a study of the table that the centrifugal force applied over a range of from 6,000 to more than 75,000 gravities makes no significant difference in the relative number of embryos found in any particular group. The lowest force used gave essentially the same result as the greatest force.

TABLE I

Eggs centrifuged before fertilization tabulated to show the force applied and the number of embryos recorded with the centripetal oil in the various possible positions in relation to the dorso-ventral axis.

Experiment Number	Force in Gravities	Time in Minutes	Oil Ventral Median	Oil Ventral Oblique	Oil Lateral	Oil Dorsal
1	6,000	3	40	35	21	4
2	6,000	6	38	34	26	2
3	6,000	12	39	30	26	5
4	20,000	3	45	35	17	3
5	50,000	3	44	36	17	3
6	50,000	5	41	36	16	7
7	50,000	12	41	35	22	2
8	75,000	5	37	31	28	4
9	75,000	5	34	33	20	5
10	77,500	3	44	27	22	7
Total per cent			41.1	33.2	21.5	4.2

It may be said that experiments Nos. 8, 9, and 10 represent the greatest forces that could be applied without bursting almost all of the eggs.

Table II tabulates similar data on eggs centrifuged at various times after fertilization and with various forces. The complete summarized data on the development and stage of centrifuging of these eggs appears tabulated in Table IV under the same experiment numbers. The percentage of the total with the oil in the ventral position is 44 per cent, with the oil ventral oblique 33 per cent, with it lateral 19 per cent. Only 4.5 per cent had the oil in the dorsal region.

It is apparent that there is no significant difference in the results depending upon the time after fertilization at which the eggs were centrifuged or between eggs centrifuged before and after fertilization. This work includes experiments carried out during the breakdown of the germinal vesicle, after both the first and second polar body formation, and just before cleavage. The influence of the centrifuging on the determination of the dorso-ventral axis is the same irrespective of the centrifugal force used.

In order to preclude the possibility that there might be some bilateral orientation in the centrifuge tubes, eggs were centrifuged in stiff gelatin before fertilization. A solution of gelatin in sea water was made that with rapid cooling remained liquid until 16° C. was reached, at which temperature the solution jelled. The eggs were put in the solution at 22° in the centrifuge tubes and cooled until the gel was quite stiff and held the eggs very firmly. The eggs were centrifuged for five minutes at 50,000 gravities, removed from the gel by breaking it up and dissolving it in sea water at 22°, and then fertilized. The position of the oil in the trochophores was recorded in two hundred embryos. In 45 per cent the oil was ventral median, in 34 per cent ventral oblique, in 19 per cent it was lateral, and in only 2 per cent was it found dorsal. These figures correspond to the results obtained when the eggs were centrifuged in a free suspension of sugar and sea water and it is concluded that there is no orientation of the eggs in the centrifuge tubes in relation to the presumptive bilateral axis.

TABLE II

Eggs centrifuged after fertilization. The complete data on the times after fertilization at which the eggs of these experiments were centrifuged are included in Table IV under the same experiment numbers.

Experiment Number	Force in Gravities	Time in Minutes	Oil Ventral Median	Oil Ventral Oblique	Oil Lateral	Oil Dorsal
12	50,000	5	45	39	15	1
13	6,000	5	41	36	17	6
14	50,000	5	42	33	16	9
16	50,000	5	44	27	22	7
17	20,000	5	48	29	20	3
20	50,000	3	44	32	23	1
Total			264	196	113	27
Total per cent			44	33	19	4.5

The influence of the centrifugal force on the determination of the bilateral axis is concluded to be the same in all experiments irrespective of the time of centrifuging before cleavage, or the force applied above 6,000 gravities. It has already been indicated in the previous section that there is no indication of a differential mortality. The centrifugal force has either induced a bilateral axis or shifted the presumptive axis. There is no evidence that a presumptive axis remains fixed and that the mortality of certain types is higher than others.

Position of the Cleavage Planes in Centrifuged Eggs

In the very large majority of eggs, centrifuged before or after fertilization, the first cleavage plane passed through the axis of centrifuging and the pole as marked by the polar bodies. In a small percentage it passed through the pole and oblique to the axis of centrifuging. In an even smaller percentage it passed through the pole and at right angles to the axis of centrifuging. The second cleavage also passed through the pole and was at right angles to the first cleavage.

It has been found by Just (1912), Morgan and Tyler (1930), Tyler (1931), and Tayler (1931) that the first cleavage has a tendency to pass through the sperm entrance point in spirally cleaving eggs. Since the first cleavage in spirally cleaving eggs bears a definite relation to the *4d* cell, and hence to the plane of bilateral symmetry, it is important to consider whether the first cleavage plane is related to the sperm entrance point in centrifuged eggs. The fertilization process of a large number of eggs that had been centrifuged was watched and the sperm was seen to enter any part of the egg and to show no preference for any particular region. Such a relation is therefore unlikely since in the great majority of eggs the first cleavage coincides with the axis of centrifuging and passes through the pole. It is statistically obvious that in most cases it cannot also pass through the sperm entrance point.

The question also arises as to whether the first cleavage plane is directly related to the dorso-ventral axis of the embryo since a small percentage of the eggs do cleave obliquely and at right angles to the axis of centrifuging. To examine this question a number of eggs were isolated in the two- and four-cell stage according to their cleavage pattern in relation to the axis of centrifuging. These eggs were centrifuged at 20,000 gravities for five minutes before they were fertilized. The results are tabulated in Table III. The number of embryos recorded is too small for statistical study, but it is quite clear that any one type of cleavage may produce trochophores with the oil in all common positions. It is concluded that in the centrifuged eggs the cleavage plane is not directly related to the bilateral axis of the embryo.

RESULTS CONCERNING DEVELOPMENT

It has already been indicated that there is normal development in the majority of eggs centrifuged before fertilization. However, that is distinctly not the case in some of the experiments on eggs centrifuged after fertilization. The developmental history of various experiments is briefly indicated in Table IV as far as it was noted. Experiments Nos. 15, 18, and 19 produced too few normal trochophores to be of value in making statistical counts of the positions of the oil in the embryos. Experiments Nos. 14 and 16 produced proportionately few normal embryos, but enough for statistical studies. In these experiments most eggs cleaved irregularly and stopped development before good morulae were formed. Experiments Nos. 12, 13, 17, and 20 produced normal trochophores from the majority of eggs. Unfortunately there are no data on the time of first polar body extrusion in Experiment No. 13, but centrifuging was probably shortly before its formation.

TABLE III

The position of the centripetal oil in relation to the bilateral axis of the trochophores is tabulated. The trochophores were reared from eggs isolated in the two- and four-cell stages according to their cleavage pattern in relation to the axis of stratification.

Trochophores	Two-cell stage		Four-cell stage		Total	Percentage
	First cleavage plane parallel to stratification	First cleavage plane at right angle to stratification	Oil evenly distributed in two of four cells	Oil in only one of four cells		
Oil ventral median	12	15	27	19	73	41
Oil ventral oblique	5	14	27	19	65	36
Oil lateral	3	9	22	5	39	22
Oil dorsal	0	0	1	2	3	2
Total	20	38	77	45	180	101

In general the abnormal development of the eggs in some of the experiments, centrifuged after fertilization and before cleavage, seems to result from interference with the maturation and cleavage spindles as Morgan and Tyler (1935) have indicated. However, these authors got very little normal development in their experiments on eggs centrifuged after fertilization.

Table IV

The data on eggs centrifuged after fertilization are tabulated as far as recorded, including the time of centrifuging in relation to the polar body formation and cleavage, polar orientation observed to have been present by the position of the oil in the trochophores, and the general viability.

Experiment Number	Time after Fertilization of Germinal Vesicle Breakdown	Time of First Polar Body Formation	Time of Second Polar Body Formation	Time of Cleavage	Time of Centrifuging after Fertilization	Centrifugal Force Applied in Thousands of Gravities and Minutes	Cleavage and Development
12	15			85	15	50Gs. 5M.	Normal development, no polar orientation since the oil is commonly found in both the anterior and post-trochal regions of the trochophores.
13				85	28	6 5	Normal development, polar orientation since the oil is commonly found only in the post-trochal regions of the trochophores.
14		30	45	85	32	50 5	Few normal trochophores developed, polar orientation.
15		40	55	90	45	20 5	Delayed and irregular cleavage, second polar body suppressed, first cleavage often into three cells. Very few survived normally.
16		30	45	85	47	50 5	Few normal trochophores developed, polar orientation.
17		40	55	90	60	20 5	Cleavage regular, normal development, polar orientation, a few exogastrulae.
18				90	70	20 5	Delayed and irregular cleavage, very few survived normally.
19		40	55	90	80	6 5	Delayed and irregular cleavage, very few survived normally, a few exogastrulae.
20				90	82	50 3	Normal development, polar orientation.

RESULTS CONCERNING POLAR ORIENTATION

It was observed in the trochophores of eggs centrifuged after polar body extrusion that the centripetal oil was almost invariably found in the post-trochal region, in the gut extending not much further towards the animal pole than the prototroch. This suggested that there might be a polar orientation of the eggs in the centrifuge tubes, a question that has been examined in part by Morgan and Tyler (1935) using lower centrifugal forces. In the present case the position of the polar bodies were determined in eggs which were centrifuged at various times after polar body extrusion. The number of eggs found with the polar bodies in these various positions is tabulated in Table V. Also a control experiment is included in which the eggs were centrifuged before fertilization.

TABLE V

The position of the polar bodies in eggs centrifuged at various times after fertilization is tabulated. The complete data on the eggs and development are included in Table IV under the same experiment numbers. The bracketed numbers express the total figures in terms of percentage for the particular experiment.

Experiment Number	Time of Centrifuging (Minutes after Fertilization)	Centrifugal Force	Polar Bodies					
			Oil Cap	Oil Cap to Equator	Equator	Equator to Third Layer	Boundary Second and Third Layers	Centrifugal Third Layer
11	Before fertilization	20,000 5 min.	21	23	—	18	—	38
17	60	20,000 5 min.	0 (0)	5 (3)	12 (8)	24 (16)	29 (19)	79 (53)
18	70	20,000 5 min.	4 (1)	48 (17)	33 (12)	62 (23)	52 (19)	75 (27)
19	80	6,000 5 min.	0 (0)	8 (9)	6 (7)	21 (24)	22 (25)	31 (35)
20	82	50,000 3 min.	0 (0)	24 (12)	29 (15)	63 (32)	49 (25)	35 (18)

For accuracy of counting only those eggs with polar bodies on or near the periphery as it appeared in the field under the microscope were counted. Temporary glycerine preparations were made and the whole slide scanned by means of a mechanical stage. Six groupings were made of the fertilized eggs, dividing the circumference of the egg into about five equal arcs and the slightly larger oil cap region.

In the group centrifuged before fertilization 44 per cent of the recorded eggs had the polar bodies above the equator in the centripetal end, and 56 per cent showed the polar bodies below the equator towards the centrifugal end. This shows little or no orientation of the pole in centrifuged unfertilized eggs and is in agreement with the results of Morgan and Tyler (1935) and Taylor (1931).

The four experiments in which eggs were centrifuged at various times after polar body extrusion all gave evidence of polar orientation on the centrifuge. This orientation seems to be rather variable at different times of centrifuging. Thus, in Experiment No. 17, centrifuged five minutes after the second polar body extrusion, only 3 per cent were found with the polar bodies above the equator, or 11 per cent including the equator, and 88 per cent had the polar bodies in the centrifugal hemisphere. On the other hand, ten minutes later (Experiment No. 18) 30 per cent had the polar bodies on or above the equator. Experiment No. 19, 10 minutes later, showed only 16 per cent with the polar bodies on or above the equator. Two minutes later, Experiment No. 20, 28 per cent were found with the polar bodies on or above the equator. A peculiar result of the last experiment is that practically no eggs had the polar bodies at the extreme centrifugal end, but the 18 per cent recorded from the centrifugal third and fourth layers in general had the polar bodies near the junction of the second layer.

TABLE VI

The position of the oil in relation to the polar axis in trochophores reared from eggs centrifuged before fertilization at various forces is tabulated and the results expressed in terms of percentage.

Experiment Number	Force in Gravities	Time in Minutes	Oil Ectodermal in Animal Hemisphere Anterior to Prototroch	Oil Endodermal in the Gut from the Vegetative Hemisphere
2	6,000	6	52	48
5	50,000	3	44	56
9	75,000	5	51	49
Total			147	153
Percentage of total			49	51

The position of the centripetal oil in relation to the antero-posterior axis of the trochophores is given in Table VI in which different centrifugal forces were used on unfertilized eggs. The oil was found in regions derived from the animal hemisphere in 49 per cent of the recorded embryos, and in the vegetative post-trochal regions in 51 per cent. The centrifugal force used made no significant difference. This result is in harmony with the result obtained counting the positions of polar bodies in eggs centrifuged before fertilization and in such eggs there is no indication of polar orientation in the centrifuge tubes.

Rough observations were made on the position of the oil in the trochophores from eggs centrifuged after fertilization. In Experiments Nos. 17 and 20, in which polar body counts had demonstrated that there was an orientation in the centrifuge tubes, the oil in the trochophores was most commonly found in the post-trochal region and rarely at the anterior end. In Experiments Nos. 13 and 14, centrifuged just before and just after the first polar body extrusion, trochophores were found to have the oil in general in the post-trochal region, indicating that these eggs also oriented in the centrifuge tubes. On the other hand, Experiment No. 12, centrifuged at the time of the breakdown of the germinal vesicle, indicated no polar orientation as the oil in the trochophores was found commonly in all regions of the embryo.

The general conclusions to be drawn from these results are that the eggs of *Urechis* do not orient in the centrifuge tubes before fertilization or following fertilization until after the breakdown of the germinal vesicle. Before the first polar body extrusion and until cleavage there is a greater or lesser tendency for the eggs to orient, in general with the pole near the centrifugal end.

Morgan and Tyler (1935) find no polar orientation in unfertilized eggs. In fertilized eggs centrifuged after the germinal vesicle breakdown and before the first polar body extrusion they find a decided tendency for the polar bodies to be extruded near the centrifugal end. They point out that this may mean a shifting of the pole rather than orientation. In three experiments centrifuging eggs after the first polar body extrusion, and one after the second polar body extrusion (data combined in their Fig. 3), they found a random distribution of the polar bodies, with no indication of orientation in the centrifuge tubes. Their method was that of centrifuging eggs in sea water in an electric centrifuge developing about 2,000 gravities. The packing effect of the eggs in the bottom of the tubes may have prevented a free rotation of the eggs, or the discrepancy between their results and mine may be due to some other more subtle reason. It is possible that at certain miotic stages there is no tendency for the eggs to orient.

These authors have also shown that in eggs centrifuged before the second polar body extrusion the second polar spindle may be driven far from the pole. In my Experiment No. 15, centrifuged five minutes after the first polar body extrusion, second polar body formation was suppressed, and the first cleavage was in general into three cells, as might be expected (Tyler and Bauer, 1937) if the spindle was driven into the interior of the egg.

CONCLUSIONS

The results of centrifuging unfertilized and fertilized eggs are essentially the same in regard to the position of the plane of bilateral symmetry in relation to the stratification. Further, there is no significant difference between experiments in which widely different centrifugal forces were used, or in which the eggs were centrifuged at widely different times after fertilization.

In 75 per cent of the total number of trochophores recorded the centripetal oil was found in the ventral or ventral oblique position. In slightly more than 20 per cent it was in a lateral position, and in less than 5 per cent it was found in the dorsal or dorsal oblique position. There can be no doubt, I think, but that *the centrifuging induces or influences the bilateral or dorso-ventral axis in the egg.* This induction is probably by the establishment of material gradients, substances concentrated in a fairly wide stratification layer at the centripetal end which influence the direction of gastrulation and determine the ventral side.

In the early work in centrifuging spirally cleaving eggs (Boveri, 1910; Conklin, 1910 and 1916; Hogue, 1910; Lillie, 1909*a* and *b;* Morgan, 1910; von Parseval, 1922; Schleip, 1914; Spooner, 1911; Tyler, 1930; Whitney, 1909; and Wilson, 1929 and 1930) embryos were obtained in some cases with the centripetal oil in any position. However, no counts were made to determine the relative numbers with the oil in the different regions, and the centrifugal forces used may well have been too small to have had any effect upon the determination of the bilateral axis. Morgan (1910), who has given the most data on this point for the egg of *Cumingia,* used a hand-turned centrifuge that developed a force of about 800 gravities. Conklin (1910) studied the effects of centrifugal forces of 600 gravities on various freshwater pulmonate eggs (*Physa* and *Lymnea*) and reported no influence on the bilateral determination. The development of the mosaic eggs of ascidians following low speed centrifuging has been studied with great care by Conklin (1931). He has found no evidence that the bilateral axis as a whole is in any way shifted by the forces used although different "organ-forming" substances have been displaced causing abnormal development.

The echinoderm egg, on the other hand, through the work of Runnström (1926) and Lindahl (1932), has been shown to develop with the bilateral axis of the pluteus definitely related to the stratification of the egg. My results (in press) of an analysis of *Dendraster* embryos developing from ultracentrifuged eggs have indicated that a predisposed dorso-ventral axis is shifted by the centrifugal forces. This analysis is based upon the fact that the amount of axial rotation induced by the centrifugal force is proportional to the force applied and is at best only a partial effect—the greater the force applied, the greater the percentage of plutei with the centripetal end in the median ventral plane, and the smaller the percentage with the centripetal end lateral. It was concluded that two factors must be involved in the bilateral determination, one factor present in the egg as a gradient and unshifted by the centrifugal force, and the other, probably diffusely distributed in the endoplasm, which is stratified towards the centripetal end to a greater or lesser extent with different forces. The plane of symmetry is then determined at the region of the greatest interactivity btween these two factors.

These results are in striking contrast with those obtained for *Urechis* eggs in which no significant difference was obtained in the relative percentages of the various described types when widely different centrifugal forces were used. It is therefore impossible to apply the type of analysis to these *Urechis* experiments that I have applied to *Dendraster*.

Because the *Urechis* experiments have given less pertinent data than the *Dendraster* experiments, at this time it seems inadvisable and impossible to attempt analysis of the factors involved in the dorso-ventral determination. There are, however, certain things that may be said.

In normal development Tyler (1931) and Tayler (1931) have shown that the sperm entrance point in general coincides with the first cleavage in *Urechis*. Assuming development as in *Thalassema* (Torrey, 1903) the first cleavage may very well then be related to the bilateral axis in normal development. However, Hörstadius (1937) has shown that in *Cerebratulus* the first cleavage may bear any relation to the bilateral axis. I have attempted to test this point in *Urechis* by vitally staining one blastomere but so far all the vital stains I have used have proved toxic.

Tyler (1931) has shown that normal trochophores may be obtained from artificially activated eggs of *Urechis*. In these experiments the sperm entrance point can play no part in the bilateral determination. In the centrifuged eggs I have already pointed out that in the majority the first cleavage cannot pass through the sperm entrance point. Nor can the sperm entrance point directly establish in these eggs the bilateral symmetry since it is random but the bilateral axis is related to the axis of centrifuging. Isolation experiments, rearing separately various cleavage types in relation to the axis of centrifuging, show that the first cleavage in these eggs is also unrelated to the determination of the dorso-ventral axis.

Hörstadius (1937), following the work of Yatsu (1910), has been unable to show any indication that the two- or four-cell stage of *Cerebratulus* possesses a bilateral predisposition. He studied the development of isolated half- and quarter-blastomeres and attempted unsuccessfully to apply the type of analysis that Hörstadius and Wolsky (1936) were able to use to demonstrate the presence of a bilateral predisposition in the early cleavage stages of *Paracentrotus*. His conclusions are that in *Cerebratulus* no apparent bilateral determination exists in the early cleavage stages.

Lillie (1909) and Wilson (1929 and 1930) have studied the development of partial eggs of *Chaetopterus* fragmented by centrifugal force. The first cleavage is found to be unequal and Wilson finds that in general it is at right angles to the stratification. Lillie concludes from the fact that the early cleavages closely resemble the normal that the bilateral axis in the uncleaved egg is present in the "ground substance" and is undisturbed by the fragmenting except in so far as an epigenetic adjustment is made regulating the size of the blastomeres to approximate the normal proportions. From such fragments Wilson has obtained quite normal dwarf larvae. He also has observed that the cleavage is dextriotropic as normal. However, since these eggs orient in the centrifuge tubes so that the polar axis comes to coincide with the axis of centrifuging, Lillie's conclusion that the bilateral axis is fixed in the egg and undisturbed by the centrifugal force seems quite unwarranted. The effect of centrifugal force on the bilateral determination of these eggs can only be studied if the eggs are held so that the polar axis is more or less at right angles to the axis of centrifuging.

It is not yet possible to say definitely that there is no bilateral predisposition in the unfertilized or fertilized egg of *Urechis*. These experiments show only that in centrifuged eggs the bilateral axis is unrelated directly to the sperm entrance point and the first cleavage plane, but is definitely influenced by the axis of stratification. It is quite clear from cell lineage studies that in spirally cleaving eggs the location of the ventral side is given by the position of the *4d*-cell and its derivatives. It seems most likely to suppose that the centrifuging induces a material gradient in the uncleaved egg that later functions to fix the properties of the *D*-cell at or near the centripetal end of the egg if the axis of centrifuging makes a considerable angle with the polar axis. It seems best to regard this bilateral determination as induced in the egg and not as a rotation or shift of a bilateral axis or predisposition already present in the egg, although such may be present in a weakly defined form.

The experiments of Hörstadius (1937) and Wilson (1929) have been particularly significant in showing that egg fragments of spirally cleaving eggs may produce dwarf larvae that seem otherwise normal. Such development cannot be called strictly "mosaic." In these experiments the dorso-ventral axis has been determined by a stratification of material substances in the *Urechis* egg. Such a modification of the normal growth pattern is also not in harmony with a strictly determinate theory of development. Bilateral determination in this egg must be regarded as indeterminant or at least quite labile.

SUMMARY

1. The eggs of *Urechis caupo* were ultracentrifuged for short periods with forces ranging from 6,000 to 75,000 gravities before fertilization and following fertilization at various times before cleavage.

2. In the trochophores 75 per cent of the total recorded embryos had the centripetal oil in the ventral median or oblique position, in slightly more than 20 per cent it was found laterally, and in less than 5 per cent dorsally.

3. The data are essentially the same for unfertilized eggs and all eggs centrifuged at various times following fertilization and before cleavage, and irrespective of the force applied.

4. From these data it is concluded that the bilateral axis of the embryo is induced or influenced by the stratification of material substances in the egg. In centrifuged eggs it is unrelated to either the sperm entrance point or the first cleavage plane.

5. There is no polar orientation in the centrifuge tubes before fertilization or following fertilization until the germinal vesicle breakdown. But following the germinal vesicle breakdown the eggs tend to orient in the centrifuge tubes so that the pole comes to lie most commonly near the centrifugal end.

6. Normal trochophores are produced from eggs centrifuged before fertilization and at certain times following fertilization, but during some miotic and mitotic stages centrifuging suppresses polar body formation and disturbs the cleavage spindles so that few normal embryos are produced.

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DEVELOPMENT OF EYE COLORS IN DROSOPHILA: NATURE OF THE DIFFUSIBLE SUBSTANCES; EFFECTS OF YEAST, PEPTONES AND STARVATION ON THEIR PRODUCTION

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In the course of our investigation of the chemical nature of the v^+ and cn^+ substances of *Drosophila melanogaster*[1] some phenotypic effects of the culture media have been discovered, which will be described in this paper. These phenotypic effects resemble those of the v^+ hormone and undoubtedly are related to its formation in a way which remains to be determined. Therefore we shall begin with an account of the present status of our chemical work[2] and then report three kinds of phenotypic effects, namely those produced by starvation, by yeast and by peptones. The obvious question of the causal relations of the mechanisms involved will be considered in the discussion.

METHODS

In our previous work on the chemical nature of the v^+ and cn^+ substances,[2] tests for the presence of these substances in a given extract were made by means of injections into animals sensitive to the corresponding substance. In the experiments reported here we used for the first time the feeding technique of Beadle and Law (1938).

Beadle and Law have demonstrated that the substances involved in the pigment formation of the wild type fly can be efficiently supplied to sensitive hosts by feeding. If vermilion brown (*v bw*) and cinnabar brown (*cn bw*) larvae are fed on cooked wild type pupae, the flies which develop show a brown (*bw*) phenotype, or a phenotype intermediate between that of *bw* and *v bw* (or *cn bw*) flies. The intensity of the effect depends on the age of the larvae at the time of the transfer: according to Beadle and Law, the best results are obtained when larvae of 60 to 85 hours after egg-laying are transferred to the food containing the active substances.

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[1] v^+ substance: a substance capable of changing the development of a vermilion (*v*) eye in such a way that it forms pigment phenotypically like that of a wild type eye.

cn^+ substance: a substance capable of changing the development of a cinnabar (*cn*) eye in such a way that it forms pigment phenotypically like that of a wild type eye.

The hormonal nature of these substances leaving no doubt, we will call them in the text either the v^+ and cn^+ substances or hormones.

[2] Earlier accounts in: Khouvine, Ephrussi and Harnly (1936); and in Khouvine and Ephrussi (1937).

On repeating these experiments with *Calliphora* extracts (*l.c.*), known to contain the v^+ and cn^+ substances, we have found that liquid extracts added to an agar solution can be tested in a similar way.

The technique used in our feeding experiments is as follows: 1 cc. of the extract or solution to be tested is mixed with 2 cc. of an agar solution. The warm mixture is poured into a cotton-stoppered shell vial, inclined at about 15° until the gelification of the medium. Unless otherwise stated, approximately thirty larvae were grown in each vial. All the experiments were performed at 25° C.

As test animals we used either apricot vermilion (w^a v) and apricot cinnabar (w^a cn), or *v bw* and *cn bw* larvae. In standard experiments the age of these at the time of the experiment was from 66 to 90 hours (after egg-laying).[3] More accurate timing was made only in some special cases, to be mentioned separately.

Our agar solution is a 3 per cent solution in Ringer's. In some of our experiments 5 per cent glucose was added to the agar solution to promote the growth of the yeast carried by the larvae.

Our standard culture medium is prepared according to the following formula: cornmeal 13 grams; brown sugar 16 grams; agar 2 grams; water 100 cc.

It will be seen that in some cases it was desirable to compare the results of the feeding experiments with those of injection. The technique of injection used was the same as previously described (Ephrussi and Beadle, 1936).

In our tables we have adopted for the description of the observed effects on *v bw* and *cn bw* flies a numerical scale essentially similar to that of Thimann and Beadle (1937). We call flies with no effect—0, and the phenotype identical with *bw*—5. One, 2, 3, 4—indicate intermediate grades of eye color modified in the direction of *bw*. In the tables the average effect (arithmetic mean) is given.

Extraction and Purification of the v^+ and cn^+ Substances

We will consider separately the extraction of the v^+ and cn^+ substances and their purification.

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[3] Unless otherwise stated, eggs were collected over approximately 24-hour periods. Slight deviations from the ages here mentioned are therefore possible.

Extraction

It has been shown (Ephrussi and Harnly, 1936; Beadle, Anderson and Maxwell, 1938; Becker and Plagge, 1937) that these substances have a wide distribution among insects and can be extracted, for example, from *Calliphora erythrocephala* larvae and pupae, which can be easily obtained in Paris. Consequently we have been able to experiment on a rather large scale.

In our earlier work we have used only the pupae and more recently both pupae and larvae. From the latter we have obtained extracts as active as the pupal extracts, although they differ somewhat in total composition.

To avoid the harmful action of oxydizing enzymes on the v^+ and cn^+ substances (referred to by Thimann and Beadle, 1937) we have used organic solvents. In our extractions we have used either of two methods, one of which gives a larger product and the other a more active product and fewer impurities although the amount of extract is considerably smaller.

Alcohol-ether Extract.—The pupae are dipped twice in liquid air for several minutes at a time. These are allowed to come to room temperature and are placed in a press. The resulting juice is shaken with twice its weight of a 1 : 1 alcohol 96 per cent-ether mixture. The supernatant liquid is decanted and the residue is pressed again. This residue with twice its weight of 85 per cent alcohol is then placed in a flask connected with a reflux condenser and subjected to boiling in a hot water bath for 20 minutes.

The liquid of the first extraction separates into two layers. The alcohol, which is the lower layer, is withdrawn and added to the second extract. The whole solution is distilled in vacuum. The remaining residue is dried with absolute alcohol and extracted with benzene. It is this residue which constitutes the *nitrogenous extract* and will be referred to later as such.

By the above method 20 grams of nitrogenous extract can be procured from 1 kilogram of pupae. This extract is highly active in 10 per cent solutions when used in the feeding test as described above, or in 1 per cent solutions when injected into the body cavity of larvae.

Carbitol Extract.—In this method the extraction was usually carried out on a mixture of pupae and larvae. The animals are crushed and dipped in three times their weight of acetone. The acetone is decanted and the residue is carefully pressed. This is crushed again and dipped in enough carbitol to cover it. It is then incubated at 29° for 18 hours, decanted and pressed. The resulting solution is yellowish brown. To this solution an equal volume of chloroform is added. If the liquid does not separate into two layers, a little water is added (however, the latter is usually unnecessary). The chloroform-carbitol layer is withdrawn and washed two or three times with water. The aqueous solutions, which still contain a little carbitol, are combined and distilled in a vacuum. The active substances are precipitated out of the remaining solution by means of ether or acetone.

In this manner we obtain what we shall name the *carbitol extract.* Six kilograms of larvae produce about 20 grams of this extract. In 0.5 per cent solutions it produces very strong effects on *v bw* flies (feeding test) and much weaker effects on *cn bw* flies. It seems that the carbitol extracts v^+ substance more nearly completely than it does cn^+ substance.

Purification of the Extracts

Purification of the Nitrogenous Extract.—A solution of nitrogenous extract is first precipitated by means of basic lead acetate; the precipitate is removed and H_2S is passed through the solution (which contains most of the substances) to remove the excess lead. The liquid is acidified with H_2SO_4 and an excess of a phosphotungstic acid solution is added. The solution is filtered and it has been found that the phosphotungstic filtrate does not contain the v^+ and cn^+ substances. The precipitate is treated with baryta and the baryta solution is found to contain these substances. By fractionating this solution with $AgNO_3$ first in acid, then in alkaline medium, the active substances are carried with the purines, the histidine, and even with the arginine.

Attempts to purify further by means of flavianic acid, picric acid, and picrolonic acid were unsuccessful, because neither the precipitate nor the filtrate were active. Apparently, the latter reagents act on the substances at this stage in a destructive manner. Reinecke salt precipitates have also been found inactive.

The substances extracted from the phosphotungstic precipitate produce weak but definite effects in a 1 per cent solution. Thus by means of this purification the activity was increased tenfold, although a large quantity of the substance was lost in the manipulation.

Purification of the Carbitol Extract.—The above-mentioned ether precipitate is dissolved in water acidified with H_2SO_4. To this, phosphotungstic acid is added until no further precipitate is formed. This is placed in the refrigerator for several hours. The precipitate is then dissolved in baryta, in which the active substances are soluble. This solution is neutralized by means of CO_2 and distilled in a vacuum. The dry residue obtained is reddish brown and is definitely active in 0.25

per cent solutions. Here again, further attempts to purify by means of picrolonic acid or the Reinecke salt have been unsuccessful.

It follows, therefore, from the above data that the v^+ and cn^+ substances are neither enzymes nor proteids. Furthermore, they are not polysaccharides, since the products of their hydrolysis do not reduce Fehling's solution. They seem to belong to the group of amino bases, since they are precipitated by phosphotungstic acid. The above conclusions are in full agreement with those of Thimann and Beadle (1937), and Tatum and Beadle (in press).

Concentration of Extracts and Intensity of Effects

Beadle (1937) has shown that wild type Malpighian tubes produce and release the v^+ and cn^+ substances. The implantation of one, two, three or four wild type Malpighian tubes into *v bw* and *cn bw* hosts has shown that the effects produced are, roughly speaking, proportional to the amount of implants.

The feeding technique offers the possibility of testing such relations in a different way. Table I gives the results of an experiment in which *v bw* and *cn bw* larvae have been fed on various concentrations of *Calliphora* nitrogenous extract. The medium used consisted of 2 cc. agar + 1 cc. of extract of which the concentration is given in the first column of the table.

Table I

Relation between concentration of extract and intensity of effect

Concentration of extract	*v bw*			*cn bw*		
	Number of hatched flies	Number of modified flies	Intensity of effect	Number of hatched flies	Number of modified flies	Intensity of effect
per cent						
0.1	8	5	0.8	4	0	0
0.5	3	3	2.0	3	3	2.0
2.5	23	23	3.0	22	22	3.3
5.0	23	23	4.5	23	23	4.0
12.5	7	7	5.0	11	11	5.0
25.0	16	16	5.0	16	16	5.0
50.0	10	10	5.0	8	8	5.0

It may be seen that, at low concentrations (0.1–12.5 per cent) the effect increases with the concentration of the extract. At 12.5 per cent the maximum effect (5 = *bw*) is obtained. This maximum is obviously reached by the *v bw* and *cn bw* flies at similar concentrations.

It should be noticed also that, except in the lowest concentration, effects are produced in 100 per cent of the flies.

The lowest concentration producing a detectable effect seems to be lower for *v bw* than for *cn bw* flies. Table II gives the results of a more detailed experiment on the action of low concentrations, which corroborates this conclusion.

TABLE II

Relation between concentration of extract and intensity of effect on v bw and cn bw

Concentration of extract	*v bw*		*cn bw*	
	Number of flies	Intensity of effect	Number of flies	Intensity of effect
per cent				
0.05	8	0	10	0
0.1	8	1.1	14	0
0.25	8	2.0	15	1.0
0.5	10	2.2	19	1.8
1.0	27	2.6	17	2.0

Experiments similar to the above have been made with agar containing glucose as well as with agar containing no glucose and with larvae carefully washed to remove the adherent yeast. The experiments gave essentially similar results. On the other hand, strong effects can be produced by adding the nitrogenous extract to the standard cornmeal medium.

EFFECT OF STARVATION

In testing the effects of some chemicals by the feeding method, it was found that some of the control animals, grown on the agar glucose mixture, showed a slight effect similar to that of the v^+ hormone. For example, in one experiment, thirty w^a *v* larvae were removed from the standard culture medium 72–96 hours after egg-laying and placed in a vial containing 2 cc. of a 3 per cent agar solution in Ringer's containing 0.5 per cent glucose. Out of 15 flies hatched in this vial, 12 hatched on the 6th day; the phenotype of these flies was normal, but the size of half of them was clearly smaller than that of the controls. Three flies hatched on the next day: one of these, although small, was normal again, and two showed an eye color clearly modified in the direction of the w^a phenotype; their size was again below the average of the control animals.

The experiment was repeated with w^a *v*, w^a *cn*, *v bw* and *cn bw* larvae and gave essentially similar results for the vermilion flies, while no effect whatsoever was observed on the *cn* flies. The experiment, later repeated on a large scale, confirmed this difference between vermilion and cinnabar flies.

The question was raised as to what part the agar or the glucose played in the production of the observed effects. Two kinds of experiments were performed: *v bw* larvae were grown either (1) on glucose-free agar, or (2) on cotton moistened with Ringer's containing 3 per cent glucose. The two experiments gave similar results: still smaller percentages of flies reached the age of hatching and among these a small number showed a modified eye color.

Special attention is drawn to the fact that only a relatively small percentage of animals showed the modification of eye color and that the flies hatched first, never showed this modification. This at once suggested that the time at which the larvae are removed from the normal food is of importance for the outcome of the experiment. This view was confirmed by experiments in which younger larvae were transferred to the agar medium. For instance, in one experiment, *v bw* larvae collected on two consecutive days were used. The age of the larvae of the first batch, at the time of the experiment, varied between 48 and 72 hours, that of the second batch between 72 and 96 hours (after egg-laying). The results of this experiment are given in Table III. As seen from the table, this experiment has shown a higher percentage of modified flies when younger larvae were treated; but the total number of flies was not modified. This suggested that the age of the larvae was not the only factor involved.

TABLE III

Relation between age of larvae and frequency of starvation effect

	Number of larvae used	Total of hatched flies	*v bw* phenotype	Modified phenotype	Percentage modified
Young larvae..........	240	29	11	18	62
Old larvae.............	240	121	82	39	32

At the time these results were obtained, it was learned that Dr. Beadle and his co-workers were concerned with a general study of the effects of starvation on the development of *Drosophila*. Our data, which needed further and more precise elaboration, were therefore communicated to Dr. Beadle. The paper immediately following this summarizes the experiments made by Beadle, Tatum and Clancy.

From the above results we conclude that undoubtedly the effects observed are due to starvation. The influence of the age of the larvae on the frequency of the effect, the variability of the size of the flies, the absence of any definite constituent of the medium to which the effect could be attributed—all point to this interpretation.

Effect of Yeast

In the course of our chemical work, when using the feeding technique, we sometimes met with the following difficulty. The fractions to be tested, mixed with agar, were often not nutritive enough to cover the developmental needs of the larvae. To overcome this difficulty, we tried to add to our media small amounts of dry yeast. Some experiments of this kind brought up an unexpected result: *v bw* flies developed with a strongly modified phenotype (modification of the eye color in the direction of the *bw* phenotype), under conditions where the effect could be ascribed only to the dry yeast.

Experiments performed for the purpose of ascertaining the nature of this relation failed at first or gave irregular results, indicating the operation of an unknown factor. Experiments have been devised then to test the possible influence of the concentration of the dry yeast. The results of these have explained the reasons for the previous inconsistency by showing that the effect, after reaching a maximum at a certain (relatively low) concentration, very rapidly decreases and falls to zero. Table IV gives the results of one of the experiments of this type (medium: 4 cc. agar + the indicated weight of powdered dry yeast). It can be seen, moreover, that while strong effects are produced on the eye color of *v bw* flies, *cn bw* show no modification whatsoever.

Table IV

Effect of different concentrations of dry yeast on v bw and cn bw

Dry yeast	*v bw*			*cn bw*	
	Number of hatched flies	Number of modified flies	Intensity of effect	Number of flies	Intensity of effect
mg.					
10	9	9	3.0	4	0
15	20	20	3.3	21	0
30	26	26	3.5	21	0
60	25	25	1.7	27	0
125	21	13	0.8	19	0
250	15	0	0	23	0

Table V gives the results of two parallel experiments on *v bw* flies, in one of which the indicated amount of dry yeast was added to 4 cc. of the agar-glucose mixture and, in the other, to 4 cc. of glucose-free agar. In the latter case the larvae, before being placed on the medium, have been carefully washed in alcohol and Ringer's to remove the adherent yeast.

It is seen that the effects are weaker in the presence of glucose. We shall return later to the question as to whether this inhibition is due to the glucose contained in the medium or to the growing yeast. In addition to the foregoing, experiments have been made with a nitrogenous extract of dry yeast, prepared in a manner similar to that of the nitrogenous extracts of *Calliphora*. Among these we will quote only one experiment, in which *v bw* larvae were grown on 2 cc. of agar (with or without glucose) + 1 cc. of extract of the concentration indicated in Table VI. Moreover, two vials of each of the media were prepared: in one of them carefully washed larvae were placed, and into the other the larvae were transferred directly from the standard cornmeal medium (Table VI).

TABLE V

Action of glucose on the intensity of the effect of dry yeast

Dry yeast	With glucose			Without glucose		
	Number of hatched flies	Number of modified flies	Intensity of effect	Number of hatched flies	Number of modified flies	Intensity of effect
mg. 10	19	14	1.5	2	0	0
15	22	21	1.9	2	2	3.3
30	23	19	1.6	17	17	2.6
60	29	9	0.3	24	24	1.7
125	35	3	0.2	23	23	0.3
250	29	0	0	22	13	0.5

We find here again that the effect increases with the concentration up to a maximum (at 2.5–5.0 per cent in this experiment) and then decreases and disappears completely; and that the effect is almost entirely suppressed in the two sets of vials containing glucose, while there is no difference between the two sets of vials containing glucose-free agar (washed and not-washed larvae). It therefore seems probable that the inhibition of the effect in the agar-glucose mixture is due to the glucose and not the growing yeast cells.

Experiments consisting of feeding *cn bw* larvae on different concentrations of dry yeast gave consistently negative results.

TABLE VI

Effect of nitrogenous extract of dry yeast on v bw in glucose-containing and glucose-free agar

Concentration of extract	Without glucose				With glucose			
	Washed		Not washed		Washed		Not washed	
	Number of flies	Effect	Number of flies	Effect	Number of flies	Effect	Number of flies	Effect
per cent								
0.25	1	0	3	0.8	14	0.1	15	0
0.5	2	0	7	0.4	20	0	18	0
1.0	14	1.4	5	0.8	18	0.3	20	0.02
2.5	20	2.1	19	2.3	21	0.1	20	0.2
5.0	16	2.6	18	2.0	16	0	20	0
12.5	17	0.9	22	0.6	21	0	20	0
25.0	14	0	20	0	21	0	22	0

Table VII summarizes the results of injection of dry yeast nitrogenous extract (dissolved in Ringer's) into *v bw* larvae. No modification of the eye color has been observed.

Since growing yeast cells are the normal food of *Drosophila*, the question was raised whether the " activity " of the dry yeast depends on its method of preparation. Two attempts to answer this question have been made.

TABLE VII

Injections of nitrogenous extract of dry yeast

Concentration of extract (*per cent*)	Number of flies	Effect
0.1	11	0
0.5	7	0
2.5	12	0

A nitrogenous extract of *fresh* yeast has been prepared. Fed to *v bw* larvae this extract has shown a fair activity (2.3) with a maximum at 2.5 per cent and a decrease above this concentration.

On the other hand, fresh yeast has been killed by freezing it twice in liquid air and melting at 38° C. Mixed with agar this yeast produced very clear effects (3.0) and also with a characteristic maximum at the concentration of 1 drop per 2 cc. agar.

From the last two experiments it cannot be concluded whether or not the living yeast cells have the same properties as the dry yeast or yeast cells killed by freezing. The question as to why the growing yeast does not produce any effect on *v* flies under the normal culture conditions cannot be answered at present. We will only mention several possible explanations: the effects described here might be produced by a property due to the preparation methods (killing, drying, extraction). On the other hand, it is possible that no effects are usually produced because the larvae ingest too high a dose of yeast. And finally the absence of effect might be due to the high sugar content of the standard culture media.

Effect of Peptones

An experiment in which thirty *v bw* and thirty *cn bw* larvae have been raised on a mixture of 2 cc. of the agar-glucose solution + 1 cc. of a 10 per cent solution of peptone (Chapoteaut), has shown that this peptone produces an effect similar to that of the v^+ hormone. Out of 21 *v bw* flies hatched, 17 showed rather weak, but definite effects (average value — 1.8). The 23 hatched *cn bw* flies did not show any modification of the eye color.

The experiment was repeated with various concentrations of peptone, the results of which are shown in Table VIII. It can be seen that, like yeast, the peptone produces an effect increasing with the concentration.

Table VIII

Effect of various concentrations of peptone on v bw

Concentration of peptone (*per cent*)	Number of flies	Intensity of effect
1	24	0
2	25	0
4	18	0.1
8	27	1.4
16	25	1.6

In the experiment given in Table IX higher concentrations of peptone have been tested. The data show that, after having reached a maximum (at 10 per cent in this experiment) the effect decreases again: here it falls to 0 at 50 per cent, i.e. the decrease is much slower than for the yeast extract.

Table IX

Relation between concentration of peptone and intensity of effect on v bw

Concentration of peptone	Number of flies hatched	Number of modified flies	Intensity of effect
per cent			
1	4	0	0
5	23	23	1.6
10	25	25	2.2
20	29	29	1.4
30	28	23	0.9
40	33	18	0.4
50	25	5	0.2

Table X gives the results of another experiment, in which 1 cc. of a 10 per cent or 20 per cent peptone solution was added either to the glucose agar mixture or to the standard cornmeal medium (test larvae —*v bw*). It is seen that the effect of peptone is suppressed in the cornmeal medium.

Table X

Comparison of the effects of peptone in agar and in cornmeal media

Concentration of peptone	In agar		In cornmeal	
	Number of flies	Intensity of effect	Number of flies	Intensity of effect
per cent				
10	22	2.2	29	0
20	26	1.7	33	0

Table XI gives the results of the comparison between three different peptones and proteoses (1 cc. of a 10 per cent solution + 2 cc. of glucose-agar; test animals—*v bw*). It appears that Witte peptone,

Table XI

Effects of various peptones and proteoses on v bw

Substance	Number of flies	Intensity of effect
Witte peptone 10%	37	2.3
Gelatin peptone 10%	35	0
Fibrin peptone 10%	37	2.1
Proteoses 10%	34	1.3

fibrin peptone and proteoses produce clear effects, while gelatin peptone does not.

It is known that gelatin peptone differs from the other peptones tested by the lack of tyrosine and tryptophane. We tested therefore on *v bw* larvae the action of these two substances alone or in combination with gelatin peptone (Table XII). The data clearly show that the mixture gelatin peptone-tryptophane produces a considerable effect. The tryptophane itself does not modify the eye color: out of 12 flies hatched in this vial only one showed an effect which we attribute to starvation.

Table XII

Complementary effect of tryptophane

	Number of hatched flies	Number of modified flies	Intensity of effect
Tyrosine 1%	11	0	0
Tryptophane 1%	12	1	0.1
Tyrosine 1%+tryptophane 1%	10	0	0
Gelatin peptone 10%	25	0	0
Gelatin peptone 10%+tyrosine 1%	25	0	0
Gelatin peptone 10%+tryptophane 1%	18	15	2.5
Gelatin peptone 10%+tyrosine 1%+tryptophane 1%	21	17	2.0

In addition to the above experiments, injections of the active mixtures into *v bw* larvae have been made. Thirty larvae were injected with a 2 per cent solution of Chapoteaut peptone; out of these four flies hatched showing no modification of eye color. Again thirty larvae were injected with a 1 per cent gelatin peptone + 0.5 per cent tryptophane mixture, out of which the eight hatched flies showed no modification of the eye color.

Mixtures of Extracts

For the comparison of the different effects studied it was of interest to see whether the "inhibiting" effect of the high concentrations of dry yeast extract will suppress the effects of active concentrations of *Calliphora* extracts or of the peptone. The results of two such experiments with *v bw* larvae are given in Table XIII. It is clear that the yeast extract has not modified the effect of a 5 per cent *Calliphora* extract, while it has completely suppressed the effect of 10 per cent peptone.

TABLE XIII

Effect of addition of dry yeast extract to Calliphora extract and to peptone

	Number of flies	Intensity of effect
Dry yeast extract 25%	33	0
Calliphora extract 5%	31	3.0
∫Dry yeast extract 25% \Calliphora extract 5%	39	3.0
Peptone (Chapoteaut) 10%	39	1.5
∫Dry yeast extract 10% \\Peptone (Chapoteaut) 10%	38	0

TIME RELATIONS IN THE EFFECTS STUDIED

In describing the feeding technique we have mentioned that, according to Beadle and Law, the effect produced on *v bw* and *cn bw* by feeding them on crushed wild type pupae depends on the age of the larvae at which this feeding is begun. Curve A in Fig. 1 gives the results of Beadle and Law for *v bw* larvae. In this curve the intensity of the effect is plotted against the age (from hatching of the eggs) at which the larvae are transferred from the standard culture medium to the tested medium. The curve of Beadle and Law shows then that the strongest effects are obtained when larvae are grown on the standard food up to 36–60 hours after hatching[5] and then transferred to the crushed wild type pupae. If transferred before or after this time, only very weak effects are produced.

The question was raised whether this time relation, characteristic of the v^+ hormone, may be found in producing similar effects by means of the dry yeast extract or the peptone. An experiment was performed in which larvae timed at hatching and grown on the cornmeal medium were transferred at various known ages to the following three media: (1) 2 cc. agar with glucose + 1 cc. of a 10 per cent *Calliphora* nitrogenous extract; (2) 2 cc. agar without glucose + 1 cc. of a 2.5 per cent dry yeast nitrogenous extract; (3) 2 cc. agar with glucose + 1 cc. of a 10 per cent gelatin peptone solution containing 1 per cent tryptophane. The results of these three experiments are given in Table XIV and Fig. 1 (curves 1, 2 and 3). We should like to emphasize that we do not ascribe to this figure any rigorous quantitative value; we merely reproduce it to facilitate the comparison of the different results.

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[5] In the paper of Beadle and Law the ages of the larvae are indicated in hours from egg-laying. The curve represented on our figure is obtained by subtracting 24 hours from the ages given by Beadle and Law. After our manuscript had been sent to press Dr. Beadle informed us that according to his determinations hatching occurs 18 hours after egg-laying. Consequently curve *A* should be shifted 6 hours to the left.

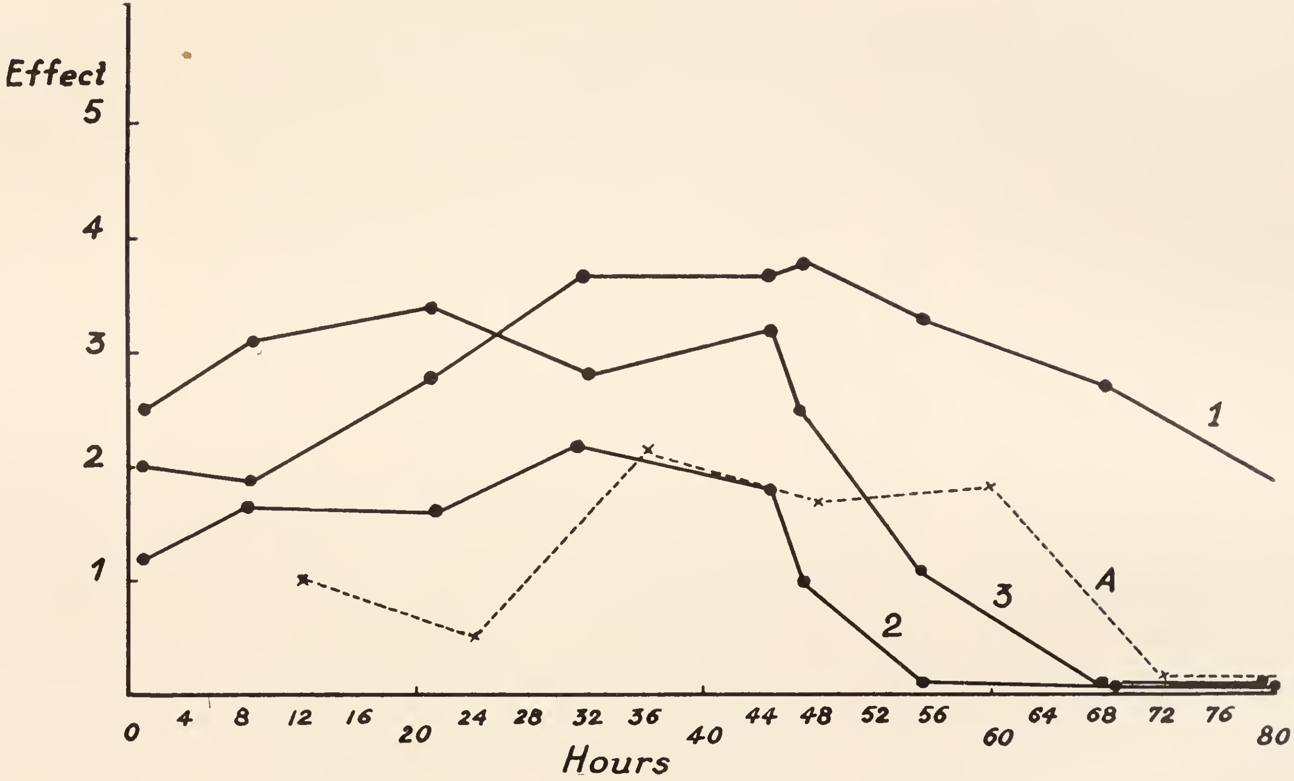

Fig. 1. Relation between effect on eye color and time of transfer of larvae (hours after hatching of eggs) from standard culture medium to the experimental medium. Curve A is based on data of Beadle and Law (1938).

As seen in the table, the age of the larvae was determined at hatching within 2–3 hours. In comparing curve 1 (*Calliphora* extract) with curve A (data of Beadle and Law) we find a fair agreement in the results. Of course the effect of our 10 per cent extract is much stronger, which explains the fact that curve 1 does not fall to 0. But then it can be seen that in both experiments the curves go through a maximum in the middle of the larval development, on both sides of which the curves fall. Special attention is drawn to the fact that the intensity of the effect produced on larvae transferred to the extract immediately after hatching is as low as that of larvae transferred just prior to pupation.

Curves 2 and 3, representing the effects produced by the yeast extract and the peptone-tryptophane solution, also show a decrease of the effect when larvae are transferred to these media after 46 hours. But there is no such clear maximum as on curve 1 and the effects produced by transferring the larvae soon after hatching from the egg are not clearly different from those obtained by the transfer at 32–46 hours. In any case the values of the effect produced by transferring at 1 hour are considerably above those produced by transferring at 68–80 hours, while curve 1 gives for these two points practically identical values.

TABLE XIV

Age of larvae at transfer (hours from eclosion)	*Calliphora* extr.			Dry yeast extract			Peptone+tryptophane		
	Total flies	Modified	Effect	Total flies	Modified	Effect	Total flies	Modified	Effect
0–2½	25	25	2.0	12	11	1.2	22	21	2.5
7–10	34	34	1.9	21	21	1.7	25	25	3.1
20–22	33	33	2.8	26	26	1.6	32	32	3.4
30–33	35	35	3.7	18	18	2.2	30	30	2.8
43½–45½	29	29	3.7	25	24	1.9	21	21	3.2
45½–48½	24	24	3.8	27	17	1.0	24	23	2.5
54–57	23	23	3.3	27	3	0.1	27	12	1.1
66–70	26	26	2.7	29	0	0	27	0	0
78½–82	25	23	1.9	27	0	0	27	0	0

DISCUSSION

In the following discussion we shall first compare the effects described above and then examine their possible explanations. Anticipating the difficulties of interpretation, we should like to emphasize that we are aware of the speculative nature of many of the arguments presented, due to the incompleteness of information in the present stage of the work. Nevertheless, a detailed discussion is judged desirable, if only for tracing the lines of new experiments.

The common characteristic of all the effects described is that they simulate the effect of the v^+ hormone. There is no reason to assume that, in the last analysis, the effects are produced by anything else than this hormone, but it is clear also that the effects of yeast and peptone (let alone starvation) are not due to the presence of hormone in these media: it has been shown that neither dry yeast, nor dry yeast extract, nor peptones produce any detectable effects when injected into the lymph of the larvae. This is, however, the crucial test for the presence of hormone as such.

Putting aside the starvation effect which is not necessarily due to the nature of the medium, we may say that the effects of peptones and of dry yeast have also in common their relation to concentration: the proportionality to it at low doses and the inhibition at higher doses. Since the inhibition is shown both by the peptone and by dry yeast and since the yeast extract can inhibit the action of the peptones, it is at least highly probable that there is no special inhibiting substance and that, on the contrary, the inhibition is bound to the active principle (whatever its nature is) contained in, or represented by, the yeast and the peptone.

It has been shown that the action of dry yeast (or yeast extracts) is inhibited by the presence of glucose in the medium. On the other hand, the effects of peptone have been observed in the glucose-agar mixture. This difference between yeast and peptone might, however, be purely quantitative. It will be noticed that the peptone action is inhibited in the cornmeal medium, characterized among other things by a higher sugar-content.

Both the peptone and yeast effects are dependent upon the age of the larvae to which they are fed. The age-effect curves are similar, although different somewhat from the curve for hormone action.

All these facts show a close resemblance between the action of the peptones and dry yeast.

Another characteristic feature of the facts described is that all the factors studied produce an effect on *v* flies and no effect on *cn* flies. This fact is a new argument in favor of the notion of Beadle and Ephrussi (1936, 1937) of the non-identity of the v^+ and cn^+ hormones. The argument is further strengthened by the fact that carbitol extracts seem to produce much stronger effects on *v*, while the nitrogenous extract produces the maximum effect at quite similar concentrations in *v* and *cn* flies.

Turning to the interpretations of the observed facts, let us first consider the possible mechanism of the starvation effect. Starvation brings about a change of eye color which is similar to that produced by the v^+ hormone. We might as well then assume that under the conditions of starvation an amount of the hormone sufficient to produce a visible effect is produced by v flies.[6] The question is then raised as to the origin of this hormone. Three different hypotheses might be suggested:

(*a*) We can assume that v flies normally produce some v^+ substance and that under the conditions of starvation the process of hormone production is accelerated more or slowed down less than other developmental reactions.

(*b*) We can assume also that v flies normally possess all the necessary elements required in the hormone formation, but that normally their metabolism is directed on a track not leading to the production of hormone. On this assumption the formation of v^+ hormone would result from the breakdown or resynthesis of some normal constituents of the tissues and, in v animals, would occur only under the conditions of starvation, i.e. they would represent a deviation from the normal metabolism.

(*c*) Finally, we might try to explain the starvation effect in terms of the quantity of food ingested. We have seen that dry yeast produces a similar effect when supplied in small amounts and that this effect is not observed when the animal is supplied with larger amounts of yeast. Assuming that living yeast has the same properties (an assumption which is by no means demonstrated) we might interpret the starvation effects as due to the ingestion of a small ("active") dose of yeast.

We will note that interpretations (*b*) and (*c*) do not imply either the complete lack, or, on the contrary, a small production of v^+ substance by v animals.

To distinguish between these three hypotheses is a rather difficult problem.

Although Beadle and Ephrussi (1937) pointed out that "from the nature of the tests used for such diffusible substances, it is obvious that 'absence' can mean only a quantity as small as or smaller than that produced by the test mutants," hypothesis (*a*) does not seem very probable on several grounds. It has been shown by Beadle, Clancy and Ephrussi (1937) that injection of v lymph into sensitive animals does not lead to a modification of eye color of the injected animals. Beadle (1937) has shown by injecting concentrated extracts of Malpighian tubes of *v* larvae (wild type Malpighian tubes do produce both the v^+ and cn^+ substances) that the amount of v^+ substance in *v* larvae is not more than about one-ninetieth of the amount present in wild type (see also Beadle, Tatum and Clancy in the following paper concerning this). We should expect then that to produce an accumulation of a considerable quantity of the so slowly produced hormone must require a very serious retardation of the development. Although small retardations always accompany a clear starvation effect, they do not appear as sufficient to account for this effect. (Precise determinations and calculations will be found in the paper by Beadle, Tatum and Clancy.)

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[6] According to a personal communication of Dr. G. W. Beadle the v^+ hormone can actually be extracted from starved v larvae.

Hypothesis (*c*) is equally hard to disprove. We will only mention here that, according to a personal communication of Beadle, the starvation effect can be observed on animals starved during a very short (sensitive) period. The total amount of yeast taken in is then very close to the normal one.

In spite of this uncertainty we favor hypothesis (*b*), which appears to us considerably supported by the well-known fact that, during starvation, the flies live mainly on their own fat bodies (Guyenot, 1917).

For the explanation of the effects of the dry yeast and of the peptones only two hypotheses remain possible, since we have already discarded the possibility that they contain the v^+ hormone:

(1) We can assume that they contain a specific substance.

(2) We can interpret their effects in terms of starvation.

It has been shown that both the dry yeast (or yeast extracts) and the peptones produce their characteristic effects only at low concentration. One might assume then that this low concentration represents a low food level and that what we referred to as inhibiting action of high concentrations is nothing but a normal nutrition. Such an interpretation, difficult as it is to discard in the case of the yeast effects, is not in agreement, however, with the observation that yeast cells grow perfectly on the peptone media used. We have to discard this hypothesis at least in so far as the peptone effect is concerned and to give our preference to hypothesis (1).

Concerning the assumption that the peptone and, possibly, dry yeast contain a specific substance, we have to make two remarks:

(1) This substance might act on the larva either directly or through the intermediary of the growing yeast. The latter interpretation does not fit, however, the effects of yeast extracts, since here the effects are observed in glucose-free media and on larvae washed to remove the adherent yeast (i.e. in the absence of growing yeast).

(2) Concerning the rôle of the substance or, more probably, substances (in view of the complementary action of tryptophane) we are,

at first sight, again in the presence of two somewhat distinct possibilities: the substances might represent normal constituents of the hormone or, on the contrary, they might have nothing to do with the hormone, except that, when taken in with the food, they change the normal metabolism of the larva in a way similar to that produced during starvation. No decisive argument for or against one of these possibilities can be advanced at present.

No conclusion as to this question can be drawn from the experiments with dry yeast extracts: while their close relation with the normal food of *Drosophila* at first sight does not incline us to think of the yeast effects in terms of a modification of metabolism, the literature contains many indications of clear biochemical differences between living and dry yeast. On the other hand, the inhibition of the yeast effect by glucose offers a striking analogy with the phenomenon of protein-sparing.

When one comes to think of the described phenomena and of the different suggested hypotheses in these terms, it becomes obvious that the different interpretations might be more closely related than they appear to be at first sight. Not only the distinction between them becomes more and more subtle, but a situation becomes perfectly conceivable in which a specific substance would produce a definite effect only in the presence of a specific deficiency. To say that an effect produced under such circumstances is due to a specific substance or to a modification of the metabolism, becomes a matter of taste. Thus, the consideration of the effects of yeast and peptone lead us to notions essentially similar to that to which we have been led by the consideration of the starvation effect. The attack of the fat bodies during starvation, the importance of tryptophane in the effect of peptones, the inhibition of the yeast effect by glucose, the conclusion of our chemical work indicating the probable amino-base nature of the v^+ substance—all point to the protein breakdown as source of this hormone.

To take in further work such an intermediate position has the advantage of drawing one's attention to the common background of the different phenomena: that of the yeast effect, that of the effect of peptones and even that of the effect of starvation.

SUMMARY

An account of chemical investigations of the nature of the v^+ and cn^+ hormones of *Drosophila* is given. It is concluded that they probably belong to the group of amino-bases.

It is shown that the effects of *Calliphora* extracts, containing these hormones, are proportional to the concentration.

It is shown that under the influence of starvation *v* flies undergo a modification of the eye color, similar to that produced by the v^+ hormone.

It is shown that similar effects are produced by feeding *Drosophila* larvae on small doses of dry yeast. At low doses the effects produced are proportional to the concentration of yeast. Higher doses of dry yeast suppress the effect which is also suppressed by the presence of glucose in the medium.

Various peptones also produce a hormone-like effect when fed to *v* larvae. Again the effect is proportional to the concentration of peptone at low concentrations, and decreases above a certain maximum.

High concentrations of yeast extract suppress the effects of the peptones, but not the effect of *Calliphora* extracts.

The relations of the age of the larvae to the intensity of the effects produced by yeast extract and by peptone are studied.

Gelatin peptone does not produce an effect comparable to that of the other peptones tested, but does if tryptophane is added.

Neither starvation, nor dry yeast, nor peptones, produce the modification of the eye color of *cn* flies.

Neither dry yeast, nor peptones produce effects on *v* flies when injected into the body cavity of the larvae.

An attempt is made to interpret all the effects observed by a single hypothesis.

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FOOD LEVEL IN RELATION TO RATE OF DEVELOPMENT AND EYE PIGMENTATION IN DROSOPHILA MELANOGASTER[1]

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Introduction

Beadle and Law (1938) have shown that the eye color hormones concerned in the differentiation of vermilion and cinnabar eye colors in *Drosophila melanogaster* are effective when administered with larval food. In attempting to develop a standardized method of feeding these hormones quantitatively, it became evident that information was needed on the relation between food-level and rate of development (see Northrop, 1917*a*, and Baumberger, 1919, for accounts of previous work bearing on this). During the course of experiments designed to give such information, Dr. Boris Ephrussi of the Institut de Biologie physico-chimique, Paris, informed us (personal communications) of feeding experiments made in his laboratory, the results of which appeared to be understandable on the assumption that food-level had an effect on pigment production in genetically vermilion brown flies. After preliminary experiments had given results consistent with this assumption, systematic studies, approaching the general problem in somewhat different ways, were undertaken in the two laboratories. The paper preceding this (Khouvine, Ephrussi, and Chevais, 1938) and the present report summarize these experiments.

The Seventy-Hour Change

If larvae are removed from standard food (cornmeal-molasses-agar, seeded with an excess of fresh baker's yeast) at various stages of development, rinsed with Ringer's solution, and transferred to vials containing filter paper moistened with Ringer's solution, there is no further increase in size. Those removed at any time up to about 70 hours after egg-laying (at 25° C., the temperature at which all experiments were carried out) may continue to live for several days but eventually die unless given additional food. Larvae similarly removed from food shortly after 70 hours fail to increase in size but continue to differentiate

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[1] Work supported in part by funds granted by the Rockefeller Foundation.

and form puparia at approximately the same time as do normally fed controls (at from 100–120 hours after egg-laying, depending on the stock used). The results of a typical series of this kind in which lots of vermilion brown larvae were removed from full food to no food at successive 4-hour intervals are summarized in Fig. 1. In this series eggs were collected over a 2-hour period; the ages indicated are measured from the midpoint of the egg-collecting period. Results similar to these have also been obtained using larvae of the Oregon-r wild type stock.

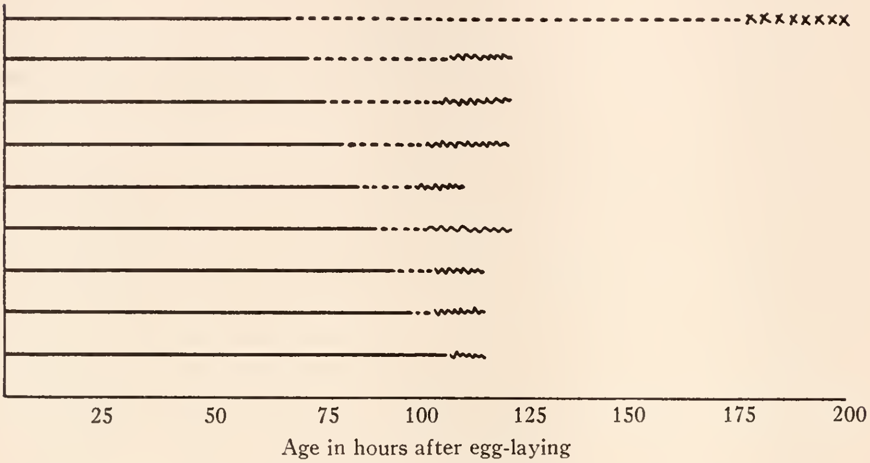

FIG. 1. The effect of removing vermilion brown larvae from food to moist filter paper at various times. Each horizontal line represents a sample of 20 individuals. Age in hours is represented on the horizontal axis. Unbroken lines represent time on full food, dotted lines time on filter paper, wavy lines puparium formation, and x's represent death.

These results show clearly that some organizational change occurs in larvae at about 70 hours (near the time of the second moult). This will be referred to as the "70-hour change" and will be considered further in subsequent sections.

RESULTS OF COMPLETE STARVATION BEFORE THE SEVENTY-HOUR CHANGE

Several series of experiments have been made in which larvae at various ages were removed from food before the 70-hour change, kept on moist filter paper for varying intervals, and then returned to standard food medium with an excess of yeast. All series, some with the Oregon-r stock, others with a vermilion brown stock, were consistent in indicating that removal from food at from 24 hours after egg-laying (some 7–8 hours after hatching from the egg) to just prior to the 70-hour change, resulted in cessation of those developmental processes concerned with differentiation. Larvae so removed from food were delayed in forming puparia, the delay being roughly proportional to the time of starvation. Since time of puparium formation was not recorded accurately (generally counts of puparia were made at 12-hour intervals), it is not considered worthwhile to record these data in complete form. The results of a typical series in which vermilion brown larvae were removed from food at 60 hours and returned in lots of 20 at 12-hour intervals are shown in Fig. 2. Other experiments included larvae initially removed at 24, 36, 48 and 60 hours, and returned in lots of 20 at 12-hour intervals up to the time of death. Under the conditions of the tests the maximum starvation periods were as follows:

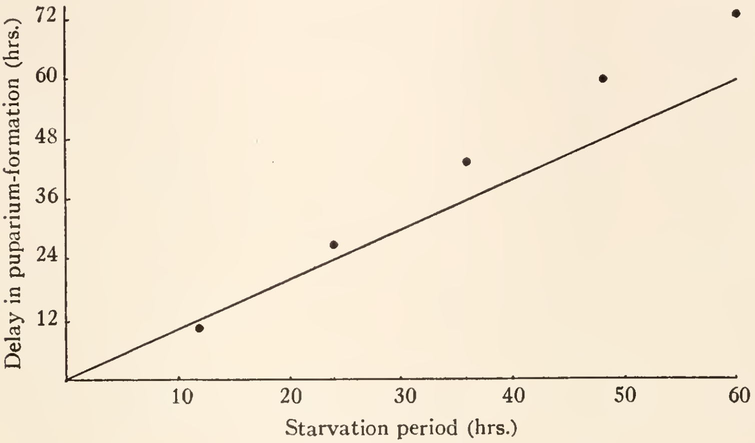

Fig. 2. Relation of delay in puparium formation to length of starvation period in vermilion brown larvae removed from food at 60 hours after egg-laying. The straight line, delay equals starvation period, is for reference.

Age at removal from food (*hrs.*)	Maximum period starved (*hrs.*)
24	48
36	36
48	60
60	60

All results were roughly similar to those shown in Fig. 2. The average delay in puparium formation in relation to time of starvation for four series of vermilion brown larvae, initially removed at 24, 36, 48, and 60 hours, is shown in Fig. 3. It is evident that the delay in puparium formation is generally greater than the time of starvation. These results suggest that under conditions of complete starvation the organizing processes which normally lead to metamorphosis are not only stopped but actually set back. Presumably materials already organized at the time of removal from food are broken down in the process of metabolism. After the restoration of an adequate food supply, these materials must be reorganized before the animal is back to the developmental stage attained at the onset of starvation. The data as obtained are not considered complete enough to justify an attempt to determine the precise relation between delay in puparium formation and time of starvation.

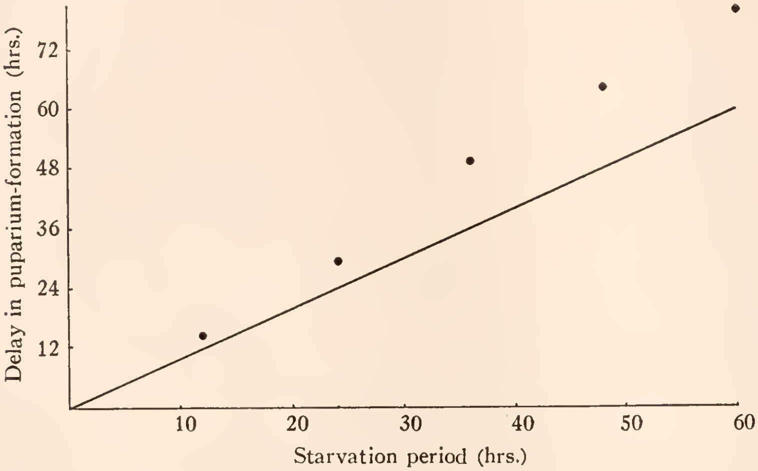

Fig. 3. Relation between average delay in puparium formation and length of starvation period, in four series of vermilion brown larvae, removed from food at 24, 36, 48 and 60 hours after egg-laying. The straight line, delay equals starvation period, is for reference.

Starvation prior to the 70-hour-change appears to be without effect on the duration of pupal life. The times from puparium formation to eclosion for four samples of vermilion brown flies subjected to periods of starvation were as follows:

Starvation period (*hrs.*)	Age at puparium formation (*hrs.*)	Time from puparium formation to eclosion (*hrs*)
None (control)	112	100
24 to 60	156	94
36 to 72	170	102
48 to 108	198	102
60 to 108	172	101

Considering the fact that both time of puparium formation and time of eclosion are approximations (observations made at 12-hour intervals), there is no evidence of any significant differences in length of pupal life. These observations agree with those reported by Northrop (1917*b*), Baumberger (1919) and by others.

Larvae subjected to complete starvation for various intervals of development prior to the 70-hour change and then returned to full food obtain full size by the time of puparium formation. Table I gives the oven-dry weights for adult flies from one series of vermilion brown larvae. It is evident that there is no consistent effect of the treatment on the final weights.

TABLE I

Weight of oven-dried adult flies from larvae subjected to starvation for various periods of development

Period starved (*hrs.*)	Total number of flies	Weight, av. ♀♀ and ♂♂ (*mg.*)
0	18	0.29
24–36	18	.26
24–48	18	.29
24–60	17	.31
24–72	7	.31
36–48	20	.27
36–60	19	.28
36–72	13	.25
48–60	19	.28
48–84	16	.34
48–96	8	.27
48–108	11	.28
60–72	19	.28
60–84	18	.26
60–96	18	.30
60–108	9	.29
60–120	3	.30

THE EFFECTS OF PERIODS OF COMPLETE STARVATION OF LARVAE AFTER THE SEVENTY-HOUR CHANGE

As indicated above, removal of larvae from food to moist filter paper after the 70-hour change does not result in a delay in puparium formation. In some series an actual acceleration of puparium formation, amounting to several hours, appeared to result from removal from food. The data obtained, considered above, are probably not sufficient to establish definitely such acceleration as a real effect of the starvation. These observations are, however, in agreement with the results of intermittent feeding on the development of the moth *Lymantria* (Kopeć, 1924). In this insect, more suitable for such studies because of the longer life cycle, starvation in late larval stages clearly brought about a hastening of pupation.

Adults from larvae removed from food shortly after the 70-hour-change are much smaller than fully-fed flies otherwise comparable. From a collection of vermilion brown larvae from eggs laid during a two-hour period, lots of 20 larvae were removed to moist filter paper at the 4-hour intervals (represented in Fig. 1). The adult flies from these larvae were oven-dried at about 110° C. until they no longer showed a decrease in weight, and their weights recorded. Males and females were weighed separately. The weight of flies (average males and females) from larvae removed from food at 72 hours was only 0.035 mg. each or only about one-eighth as much as fully fed controls which weighed 0.290 mg. each. The relation of final weight to time of removal from food is shown in Fig. 4. It is clear that size as determined by weighing is roughly proportional to the time after the 70-hour-change during which the larvae are allowed to feed. Whether or not

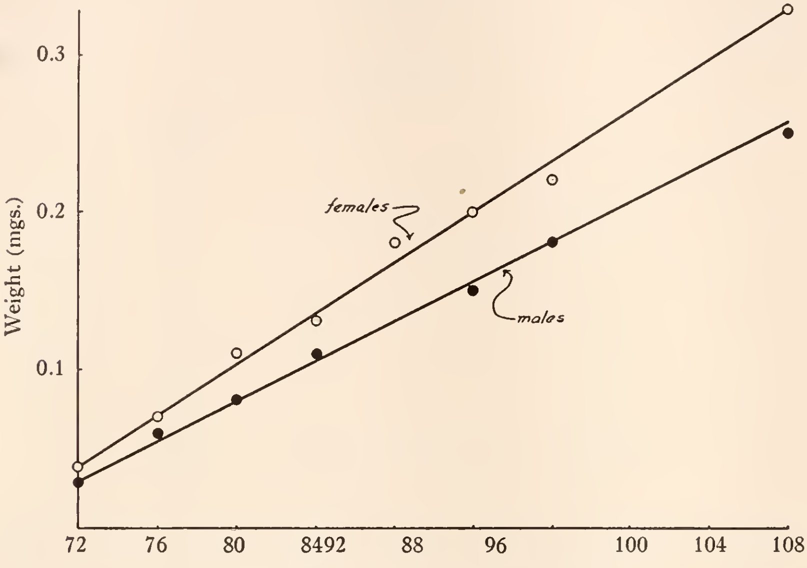

Fig. 4. Relation between oven-dry weight of adult flies from vermilion brown larvae removed from full food at successive 4-hour intervals and allowed to complete development on moist filter paper.

the relation is linear, as Fig. 4 might suggest, the data are not sufficient to determine.

Aside from their size, these flies, starved from 72 hours, appeared to be normal. Tests made by intercrossing the small flies resulting from complete starvation of larvae after 72 hours showed that both sexes were fertile; no attempt was made to mate such small flies to flies of normal size.

In general, the same effects of starvation as are considered above in *Drosophila* were observed in *Lymantria dispar* by Kopeć (1924). Kopeć discusses in some detail the significance of such results as these to the more general problems of development and differentiation.

ATTEMPTS TO INDUCE THE SEVENTY-HOUR-CHANGE

On the assumption that specific organizing substances might be concerned in the 70-hour change, extracts of animals that had undergone this change were injected into larvae removed from food before 70 hours. Wild type larvae, taken shortly before puparium formation, were ground with powdered silica in an ether-alcohol mixture. The liquid was decanted, evaporated by gentle warming, the residue taken up in water, and centrifuged. The oily extract obtained gave negative results when injected into vermilion brown larvae removed from food at 68 hours (15 test larvae died without forming puparia). The water extract obtained by the above procedure was similarly tested, and of 15 test animals, one pupated. In the uninjected control animals, however, one in 45 also pupated, so that there was no definite indication of a positive effect.

Tests were made of fluid obtained by crushing and centrifuging wild type pre-pupae with white puparia, i.e., larvae that have formed puparia within an hour. The water layer was negative when tested in vermilion brown larvae taken from food at 68 hours. The results were the same with fluid from unheated and heated (100° C. for about 1 minute) pre-pupae (12 tests with heated and 14 with unheated material).

Preliminary experiments indicated that a ring gland (Hadorn, 1937) from a 100 ± hour larvae transplanted to larvae removed from food at 68 hours would induce puparium formation and pupation at the time that fully fed controls underwent these changes. Additional tests of the same type gave results that were not convincing. Of sixteen 68-hour test animals injected with ring-glands from 100 ± hour vermilion brown larvae, 15 lived and of these 6 formed puparia but did not continue to develop. Of a similar number of surviving controls injected with Ringer's solution, 2 formed puparia and likewise failed to continue development. In another test, 3 of 10 animals to which ring glands had

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been transplanted formed puparia and failed to continue development. In this test all 17 controls injected with Ringer's solution failed to form puparia. It appears from these results that the ring gland may exert some influence on organization processes, but this is by no means proved. Furthermore, assuming such an effect, the possibility has not been excluded that it is simply a question of adding more potential food material; that is, the addition of a ring gland might be roughly equivalent to feeding the larvae for a few hours.

Clearly more information is needed before an attempt to interpret the nature of the 70-hour change in specific terms is likely to be profitable.

Relation of Food Level to Eye Pigment Development in Vermilion Brown Flies

Preliminary trials showed that by taking larvae of apricot vermilion or vermilion brown flies and placing them under conditions of reduced food, a darkening of the eyes could be induced. The experiments reported below, all made with vermilion brown flies, were designed to find out more about this starvation effect.

Conditions appropriate for producing a modification in the eye color of vermilion brown flies have no detectable effect on the eye color of cinnabar brown flies.

Effects of Complete Removal of Food

In the series of experiments reported in previous sections of this paper, in which vermilion brown larvae were removed from food to filter paper at different ages and completely starved for varying lengths of time, careful observations were made of the eye colors of the adult flies obtained. The results were consistently negative. Complete removal from food was without apparent effect on the eye color regardless of the age of the larvae at the beginning of the starvation period and of the duration of the starvation period. Intermittent feeding gave similar negative results. In one such series 44 larvae, initially removed from food at 36 hours after egg-laying, were treated as follows:

Time on food (*hrs.*)	Time off food (*hrs.*)
Hatching to 36	36 to 72
72 to 88	88 to 109
109 to 121	121 to 135
135 to 216 (puparium formation)	

With this treatment, purposely made drastic, the mortality was high; only two flies survived. These showed no modification of the eye color.

Other experiments of essentially the same nature likewise gave negative results.

Effects of Intermediate Food Level

It was found in one experiment that vermilion brown larvae completely starved from 48–96 hours, then fed a few drops of yeast suspension on filter paper, as the yeast became exhausted gave rise to adults with eyes definitely darker than the controls. Four flies eclosed on the twelfth day after egg-laying were only slightly modified, 13 that emerged on the thirteenth day gave a maximum color value of 1.0 (0.0 represents no modification, 5.0 a complete change to brown; see Tatum and Beadle, in press, for details concerning eye color standards). This and similar experiments suggested, then, that an intermediate food-level was effective in inducing a modification of the processes concerned with eye pigment formation.

To determine the possible influence of varying food levels on the eye color of vermilion brown flies, vials were made up containing 10 cc. of 1 per cent brewer's yeast in 2 per cent agar. Varying numbers of larvae were removed from full food at 25 hours after egg-laying and placed in such vials. It was found that mortality increased as food level decreased, from 0 per cent with 10 larvae per vial to 50 per cent with 100 larvae per vial. Delay in puparium formation increased as the amount of food decreased, and, in general, size of adult flies decreased with decrease in food available per larva. The effect on eye color was marked, as indicated in Table II. A general relation was observed between size of adults and strength of modification; i.e., smaller flies were darker.

The data in Table II suggest that there is a food level between the two extremes represented in the experiment at which the modification in eye color is at a maximum. That there should be such an optimum food level for inducing this response is, of course, obvious from the fact that the response can be induced and that the two extremes, full food and no food, are ineffective.

A second experiment of the same nature but using 10 cc. of 1 per cent agar containing 0.5 per cent brewer's yeast, gave essentially similar results. As expected, the larval mortality was higher, dropping from 20 per cent with 10 larvae per vial to 80 per cent with 100 larvae.

Period of Development Sensitive to Low Food Levels

In order to bring about a modification of the eye color of vermilion brown flies by means of reduced food, the larvae must be put at a low food level before a certain period of larval development. Table III

Table II

Results of different food levels on the eye color of vermilion brown. Varying numbers of larvae were transferred at 25 hours after egg-laying (7–8 hours after hatching) to vials containing 10 cc. of 2 per cent agar agar with 1 per cent of dry brewer's yeast. Results are recorded in arbitrary color values followed in parenthesis by the number of individuals on which the color value is based. Fully fed controls showed no modification of eye color.

Number of larvae per vial	Delay in eclosion as compared with fully fed controls—days				
	1	2	3	4	5
10 20 30*	1.6 (42)	2.8 (7)			
40*	1.9 (19)	3.2 (4)			5.0 (1)
50*	1.9 (17)	2.1 (7)	4.3 (3)	4.2 (2)	
100		2.0 (18)			

* Thirty flies from the 30-, 40-, and 50-larva vials were combined. These eclosed 2 days later than controls and gave a color value of 2.8 (2.0–4.0).

gives the results of an experiment in which vermilion brown larvae of various ages were removed from full food to a low food level. Larvae transferred at any time up to and including 63 hours after egg-laying showed a relatively strong modification of eye color. Transferred at 75 hours or later, the result is quite negative. Within the limits of the experiment, this period, after which the eye color change no longer results, coincides with the 70-hour change. This result was confirmed in a second series not reported here in detail.

Table III

Effect on eye color of removing vermilion brown larvae from full food at different ages and placing them on a low-food level. The low-food medium consisted of 10 cc. of 2 per cent agar containing ¼ per cent of air-dried brewer's yeast per vial. Twenty larvae were placed in each vial. Ages are measured from egg-laying.

Age at transfer to low food	Age at return to full food	Number of adult flies	Color value, mean and range
(*hrs.*)	(*hrs.*)		
28	175	11	3.7 (2.8–4.5)
39	175	19	3.4 (2.0–4.0)
51	175	10	2.7 (1.0–3.5)
63	175	14	3.3 (2.0–4.5)
75	Not returned	20	0.0
87	Not returned	18	0.0
99	Not returned	19	0.0
Control		17	0.0

The transfer to low food appears to be no more effective if made early in development than if made at 63 hours (Table III). In other words, it appears that the period primarily sensitive to the treatment lies between the ages of 63 and 75 hours. In order to test this further, experiments were made in which vermilion brown larvae transferred to low food about 10 hours after hatching from the eggs were returned to full food at intervals. The results of such an experiment are presented in Table IV. Subjection to a low food level early in development has very little effect. If the larvae are left on the low food medium until 87 hours after egg-laying the modification is very much stronger. Of course this chronological age of 87 hours does not correspond to a similar developmental age. By subtracting from the chronological age the delay in puparium formation we get a rough approximation of the developmental age. It is found by doing this that, within the limits of accuracy of the experiment as carried out, this type of experiment gives results consistent with the reciprocal one just described; that is, to obtain marked modifications of eye color, larvae must be subjected to a low food level during a period corresponding to that in the region 60 to 70 hours of normal development.

TABLE IV

Effect on eye color of returning vermilion brown larvae grown at a low food level (as described in heading to Table III) from 28 hours after egg-laying to full food.

Age at return to full food	Approximate delay in pupation	Number of adult flies	Color value, mean and range
(*hrs.*)			
39	8	10	0.0
51	15	17	0.2 (0.0–0.5)
63	18	12	0.2 (0.1–0.3)
75	18	17	0.3 (0.2–0.5)
87	24	14	1.8 (0.2–2.5)
99	36	14	0.7 (0.1–2.0)
Control	Full food throughout	17	0.0

If larvae are kept on full food up to 60 hours after egg-laying, transferred to a low food medium and returned at intervals to full food, it is found that the modification in eye color increases with increasing time on low food. The results given in Table V show this. The maximum effect is produced in the lot of larvae kept on low food for 66 hours. The delay in puparium formation and eclosion (only roughly measured) indicates that during this time they had advanced developmentally some 16 hours or less.

The above results are in general agreement in showing that the period sensitive to low food level as measured by the production of eye pigment lies between the stage reached at about 60 hours at full food and the 70-hour change, and that the maximum response is obtained when all of this developmental period is gone through on a low food level.

Relation of Food Level to the Eye Color Hormone System

Since it is known that the addition of a hormone-like substance to a vermilion fly will result in the production of an eye color like that of wild type (see Ephrussi, 1938, for review and references to literature), an attempt was made to determine whether or not the increase in eye color intensity caused by partial starvation resulted from the production of increased amounts of v^+ hormone by such flies. Approximately 1700 vermilion brown larvae were grown under conditions calculated to give a reasonably strong modification in eye color. These were allowed to pupate and some 20–60 hours after puparium formation they were collected, cleaned and oven-dried at 100° C. In this way 0.69 gram of dry material was collected. A sample of 9 such flies allowed to develop gave a mean color value of 2.3 (1.5–3.0). As a control 4.5 grams of oven-dried vermilion brown pupae, fully fed as larvae, were collected. A sample allowed to develop gave a color value of 0.0 as expected. Crude extracts of these two lots of material were prepared according to the method described by Tatum and Beadle (in press). This extract in

TABLE V

Influence of length of time on low food level on the eye color of vermilion brown flies the larvae of which were transferred from full food to low food at 60 hours after egg-laying. Low food has the same significance here as in Table III.

Age at return to full food	Time at low food level	Number of adult flies	Color value, mean and range
(*hrs.*)			
69	9	19	0.0 (0.0–0.5)
77	17	17	0.1 (0.0–0.4)
84	24	18	0.7 (0.0–3.3)
94	34	15	1.3 (0.4–1.7)
101	41	13	0.8 (0.4–2.0)
126	66	10*	3.1 (1.0–4.3)

* Approximate delay in eclosion as compared with controls, 50 + hours.

a rather concentrated form was tested in the usual way by injection into vermilion brown test animals. From the tests of the extract of the starved vermilion brown pupae, it can be calculated that at the same concentration as the non-starved control this extract would have given a maximum color value of 1.6 (range 0.8–1.6). The reduced food level therefore resulted in an increased production of v^+ hormone by vermilion brown flies. From the results reported by Tatum and Beadle, we can calculate that an extract of wild type pupae of a concentration equal to that of the non-starved vermilion brown pupae would contain about 112 units of hormone per injection. Since one-third of a unit per injection can be detected without difficulty, the hormone content of the non-starved vermilion brown pupae can be said to be less than one three hundred and thirty-sixth that of wild type. The amount of hormone extracted from the partially starved vermilion brown pupae amounts to approximately 14 per cent of that extractable from wild type pupae. The relation between eye color and amount of hormone available per fly, given by Tatum and Beadle, indicates that at least 30 units are normally produced by a wild type fly. The extraction method used yields about 10 units per wild type fly. Assuming this same ratio of amount extracted to the amount produced to hold for the vermilion brown flies grown on reduced food, we can calculate that these flies would have produced an average of about 4.2 units per fly. This corresponds to an eye color value of 2.7 which is as close as could reasonably be expected to the observed color value of 2.3.

The magnitude of the increase in v^+ hormone resulting from reduced food can be approximated in the following manner: assuming that a vermilion brown fly does produce some v^+ hormone, we know that this amount must be less than one-third of a unit as defined by Tatum and Beadle. The maximum effect produced by growing vermilion brown larvae on a low food level is a color value of 5 which corresponds to an injection of at least 30 units of hormone. It follows, therefore, that the hormone is increased at least a hundredfold by subjecting vermilion brown larvae to the appropriate food level.

DISCUSSION

From the results presented above it is seen that the conditions resulting in what might be called the "starvation effect" on the eye color of vermilion brown flies are accompanied by a retardation in development. That this retardation is, in itself, not responsible for the effect is shown by the fact that larvae retarded by complete removal from food do not show it. Furthermore, experiments not reported above have shown that retardation during the sensitive period brought about by subjecting larvae to a temperature of 15° C. is quite without effect. The final size attained by the fly has nothing essential to do with the effect since larvae returned to full food after the effective period of the treatment are normal in size and may show strong modification of eye color.

A possible explanation of the effect of food level on the eye color change that we are concerned with involves the assumption that the effective low food levels may affect developmental reactions differentially. It might be supposed, for example, that the reactions leading to the formation of v^+ hormone go on slowly in a vermilion fly and that by prolonging development other reactions may be retarded and at the same time the conditions remain favorable for the formation of v^+ hormone. The magnitude of the effect, however, is such that this interpretation seems improbable. As pointed out above, the increase in hormone is at least one hundred times that in fully fed vermilion brown flies. With no change in rate, we would then have to assume an increase in time during which a reaction goes on of at least this magnitude. Since the delay accompanying the maximum response is less than 100 hours, the normal time required for the reaction assumed to be prolonged by the treatment would be less than one hour. While this simple interpretation, of course, is conceivable, it does not seem probable, especially in view of the fact that the sensitive period appears to extend over a period corresponding to about 10 hours of normal development.

Although the mechanism by which the starvation effect is brought about is unknown, it is suggested, as an hypothesis, that the food level effective in bringing about the eye color change modifies metabolism in such a way that the products normally used in one manner in a vermilion fly are utilized in the formation of v^+ hormone. The hormone involved is known from the work of Khouvine and Ephrussi (1937) and that of Tatum and Beadle (in press) to be amino acid-like in nature. It seems reasonable, therefore, to suppose that amino acids of one kind or another are the precursors of this hormone and that conditions are such in a vermilion fly that these precursors are utilized largely in protein synthesis rather than in the production of hormone. The conditions of low food that result in the formation of an appreciable amount of hormone are such that protein breakdown, localized or general, might be expected to result, and it seems possible that intermediate products of this breakdown, possibly amino acids, could be diverted to reactions leading to hormone synthesis. For further discussion of this question see the accompanying paper by Khouvine, Ephrussi and Chevais.

On first thought it seems strange that the starvation effect should not have been noted previously in stock bottles. It is, of course, a well-known fact that under the normal conditions of *Drosophila* culture, the larvae are not optimally fed. Observations of crowded stock bottles made for the express purpose of determining whether or not vermilion brown flies showed the modification under these conditions, were negative. Presumably under standard culture conditions the drop in the amount of food available is rapid with the growth of larvae and the intermediate level at which the effect becomes strong is maintained for a relatively short time. The experiments reported above show that the appropriate food level must be maintained for some 36 hours and that this level must coincide with the sensitive period of development. Thus it appears that the conditions that result in the starvation effect are not likely to be met in standard culture techniques.

It is evident from the results reported here and in the preceding paper by Khouvine, Ephrussi and Chevais that the detection or measurement of v^+ hormone by the feeding technique may be complicated by the so-called starvation effect. From the fact that larvae older than 70 hours (full food at 25° C.) no longer show the eye color change associated with reduced food, but still react to v^+ hormone in the food medium, it is clear that feeding tests made with larvae transferred from full food after 70 hours are not subject to this complication.

SUMMARY

In the development of larvae of *Drosophila melanogaster* an organizational change takes place at about 70 hours after egg-laying (25° C.). This change is referred to as the "70-hour change."

Larvae deprived of all food for periods at any time up to the 70-hour change are retarded in development, the retardation being a function of the length of the starvation period.

Larvae deprived of food after the 70-hour change are not retarded, but are probably somewhat accelerated in development.

If deprived of food during a portion of the larval developmental period after the 70-hour change, the final size attained by the fly appears to depend largely on the proportion of this period spent on full food.

Periods of complete removal of food from vermilion brown larvae are without apparent effect on eye pigment production. This is true whether the starvation period be short or long and whether it comes before or after the 70-hour change.

Partial starvation (at intermediate food levels) of larvae, under certain conditions, greatly increases the amount of eye pigment formed by vermilion brown flies.

The period of development most sensitive to this " starvation effect " on pigment production by the eye, lies shortly before the 70-hour change.

The production of a supposedly specific eye color hormone, v^+ hormone, can be influenced greatly by the amount of food given to vermilion brown larvae during a limited period of development, the period of maximum sensitivity referred to above. This increase is not less than a hundredfold.

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STUDIES ON TREMATODES OF WOODS HOLE: THE LIFE CYCLE OF LEPOCREADIUM SETIFEROIDES (MILLER AND NORTHUP), ALLOCREADIIDAE, AND THE DESCRIPTION OF CERCARIA CUMINGIAE N. SP.[1]

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The results reported in this paper were obtained during the summers of 1936 and 1937 at the Marine Biological Laboratory at Woods Hole, Mass. They include a possible life cycle of *Lepocreadium setiferoides* (Miller and Northup, 1926) and the description of a new species of trematode, *Cercaria cumingiae,* with observations on its life cycle. In the adult stage, *Lepocreadium setiferoides* parasitizes the flounder and hence may be of some economic importance.

Material and Methods

The various hosts of the trematodes described in this paper were collected near Woods Hole. The mollusk, *Nassa obsoleta,* the first intermediate host of *L. setiferoides,* was secured at Quisset Harbor. The second intermediate hosts of this trematode, the turbellarian, *Procerodes warreni,* and the annelid, *Spio* sp., were collected at Nobska Beach and Sheep Pen Harbor. The flounders and sand dabs, that serve as hosts of the adult worms, were collected from various shallow waters. *Cumingia tellinoides,* which serves as both the first and second intermediate hosts of *Cercaria cumingiae,* was collected from a number of localities.

The mollusks were isolated in finger bowls and examined under the dissecting microscope for the emergence of cercariae. When an infected *Nassa* was found, the second intermediate hosts were placed with it in a finger bowl of sea water. After an interval of several days, the infected second intermediate hosts were fed to flounders and sand dabs. *Nereis* was used as a food supply for the fishes since it was found that this worm would not serve as a second intermediate host of *L. setiferoides.*

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[1] This work was done under the direction of Dr. R. M. Cable to whom my thanks are hereby expressed.

Neutral red was used in the study of living material, particularly to stain glandular tissues. Living material was flattened under light cover-glass pressure and fixed in a saturated aqueous solution of mercuric chloride. Mayer's paracarmine was used to stain toto-mounts.

OBSERVATIONS

The life cycle of *Lepocreadium setiferoides* includes the development of the larval stages in *Nassa obsoleta,* the encystment of the metacercaria in the turbellarian, *Procerodes warreni,* and the annelid, *Spio* sp. and the development of the adult in the sand dab and flounder.

The Redia (*Fig. 2*)

The rediae develop in the digestive gland of *Nassa obsoleta.* They are elongate, with bluntly rounded anterior end and pointed posterior end. A collar and ambulatory processes are lacking. No birth pore was observed. In flattened, fixed specimens, the rediae vary in length from 0.43 mm. to 0.94 mm.; in width from 0.088 mm. to 0.11 mm. The pharynx measures approximately 0.037 mm. in length by 0.040 mm. in width. Young rediae contain as many as thirty germ balls but no mature cercariae. A sac-shaped gut extends posteriorly from the pharynx as far as one-third the length of the redia.

The Cercaria (*Fig. 3*)

The cercariae possess two conspicuous eye spots near the level of the pharynx. The tail is provided with lateral tufts of setae.

Observations and measurements of living cercariae under light cover-glass pressure are as follows: the body is 0.305 mm. long by 0.10 mm. in maximum width at the level of the ventral sucker; the tail is 0.576 mm. long by 0.047 mm. wide at its proximal end; the caudal setae are 0.073 mm. long at the proximal end of the tail and somewhat shorter distally. The setae are arranged in transverse rows, of from four to seven setae each, on either side of the tail. Miller and Northup (1926) stated that there were about thirty pairs of setae groups on the tail, but in the writer's specimens, thirty-five pairs of tufts were found in addition to an unpaired group of setae at the tip of the tail. The bases of the setae are expanded and fused with one another. The tail is composed of an outer sheath of muscles and an inner vacuolated, parenchymatous core. The cuticula of the body is provided with small spines which decrease in number towards the posterior end of the body. The body varies in shape with the degree of contraction. The oral sucker is about 0.058 mm., the ventral sucker about 0.044 mm. in diameter. The two conspicuous, black eye spots are about 0.015 mm. in diameter and are located approximately midway between the two suckers. The prepharynx varies in length with the degree of contraction of the body, generally being very short. The pharynx measures 0.022 mm. in length by 0.015 mm. in width. The esophagus is approximately as long as the pharynx. The intestine is rudimentary, sometimes appearing as two strings of cells, staining deeply with neutral red, extending from the esophagus to near the posterior end of the body. Eight cephalic glands are present on each side of the body and partially surround the ventral sucker. Their ducts pass anteriorly and open to the exterior at the anterior end of the body. These glands stain deeply with neutral red but in the same general region there appear to be other glands that do not absorb this stain. From ten to fifteen cystogenous glands are scattered irregularly through each half of the body.

The reproductive system is well developed. The two testes have slightly lobed or smooth margins and are located in the posterior one-third of the body. The ovary lies slightly to the right of the mid-line between the anterior testis and the ventral sucker. The accessory reproductive structures are not readily distinguishable.

The excretory bladder is a conspicuous, tubular sac which extends from the posterior end of the body to a short distance in front of the ventral sucker. It contains numerous, refractile, discoid concretions whose lines of accretion may be clearly seen under the oil immersion lens. At the posterior end of the bladder there is a muscular sphincter about 0.006 mm. long by 0.005 mm. wide. The excretory pore opens dorsoventrally. The ciliated, main collecting tubes arise from the excretory bladder at the level of the testes and pass cephalad as far as the level of the anterior margin of the ventral sucker before each divides into an anterior and a posterior secondary collecting tube which supply their respective regions of the body. The secondary tubes divide into capillaries which end in flame cells. There are 24 flame cells on each side of the body which appear to be arranged in groups of threes. However, one of the capillaries of each group joins the secondary collecting tube independently of the other two.

The cercariae emerge both diurnally and nocturnally and are photonegative. They swim rapidly about by lashing the tail.

The Metacercaria

The cercariae were observed to penetrate and encyst in the triclad turbellarian, *Procerodes warreni,* and in spionid worms. The tail is shed in the process of penetration. These experimental hosts may not be the natural ones, particularly *Procerodes* which is found in rocky regions which are not the habitats of the flatfish. The metacercaria does not increase much in size but the reproductive systems become more mature.

The Adult (*Fig. 1*)

Natural infections of *Lepocreadium setiferoides* were found in most of the small flounders and sand dabs so that suitable experimental animals, known to be uninfected with this parasite, were not available. However, the flounders were fed infected *Procerodes* and spionids over a period of about one month and various stages of development from the metacercarial to the adult were obtained. The following description and measurements are based upon observations of 35 preserved worms. The cuticula is covered with scale-like spines about 0.005 mm. long at the anterior part of the body but somewhat smaller at the posterior end. The body length varies from 0.35 mm. to 0.58 mm. with an average of

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EXPLANATION OF PLATES

All drawings except Fig. 7 were made with the camera lucida.

Abbreviations used:

C	cephalic gland
CG	cystogenous gland
CS	cirrus sac
E	esophagus
EB	excretory bladder
EC	excretory concretion
EG	egg
ES	eyespot
F	flame cell
G	genital primordium
GB	germ ball
I	intestine
L	lappet
O	ovary
OS	oral sucker
P	pharynx
PP	prepharynx
SE	seta
SP	sphincter
T	testis
V	vitellaria
VR	vitelline reservoir
VS	ventral sucker

Description of Plate I

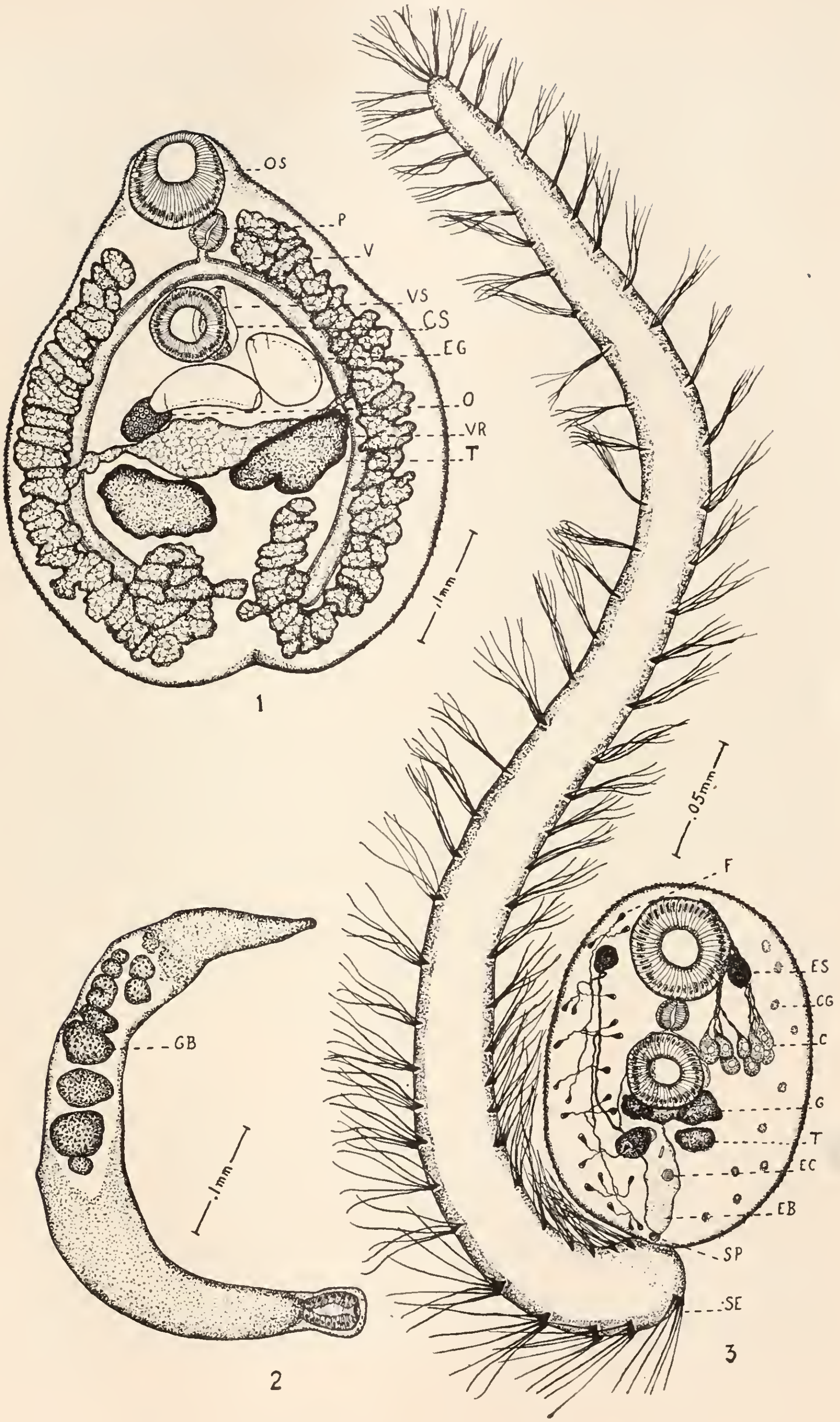
All figures concern *Lepocreadium setiferoides.*

Fig. 1. Ventral view of adult.

Fig. 2. Young redia showing germ balls.

Fig. 3. Ventral view of cercaria.

PLATE I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0.43 mm. The body width varies between 0.19 mm. and 0.35 mm., averaging 0.26 mm. The oral sucker is approximately circular and averages about 0.077 mm. in diameter. The mouth opens into a very short prepharynx. The pharynx averages 0.045 mm. in length by 0.043 mm. in width. The intestine bifurcates in front of the ventral sucker and the intestinal ceca extend to near the posterior end of the body.

The vitellaria extend from the anterior margin or the middle of the pharynx to the posterior end of the body where they may become confluent. Vitelline ducts carry the yolk material to a reservoir located near the ovary. The ovary is located to the right of the mid-line of the body between the anterior testis and the ventral sucker. It is oval in shape and averages about 0.04 mm. by 0.044 mm. A short Laurer's canal opens to the exterior on the dorsal surface of the body. The seminal receptacle is spherical in shape and approximates the size of the ovary although its size varies considerably. The uterus coils between the anterior testis and the ventral sucker to open at the genital pore to the left of the anterior margin of the ventral sucker. The eggs are few in number and very large in proportion to the size of the worm. Their length varies from 0.05 mm. to 0.11 mm., averaging 0.066 mm.; their width varies from 0.04 mm. to 0.073 mm., with an average of 0.07 mm.

The two testes are obliquely arranged in the posterior half of the body. They are oval in outline with smooth or lobed margins. The right testis averages 0.066 mm. by 0.076 mm. while the left testis averages 0.07 mm. by 0.074 mm. The cirrus sac is oval in outline and extends from the genital pore to a point slightly posterior to the ventral sucker. The cirrus is tubular and slightly coiled. The prostatic cells are few in number. A seminal vesicle is located in the posterior part of the cirrus sac and in a few worms slight dilatations of the ductus deferens were observed external to the cirrus sac.

Discussion

Miller and Northup (1926) described the cercarial stage of this trematode as *Cercaria setiferoides*, stating that it was similar to *C. setifera* Müller, 1850 (described by Monticelli, 1914, and redescribed by Odhner, 1914). Palombi (1932, 1934*b*) has shown that Monticelli described several setiferous-tailed cercariae under the name, *Cercaria setifera*. A number of workers, including Villot (1875, 1879), Fewkes (1882), Huet (1891), Giard (1897), Odhner (1911), Dollfus (1925), and recently, St. Markowski (1936) and Hopkins (1937), have described setiferous-tailed cercariae. Some of these larvae have Y-shaped excretory bladders and probably belong to the genus *Bacciger* as shown by Palombi (1934*a*). The setiferous-tailed cercariae described by Hopkins develop into adults belonging to the genera *Anallocreadium* and *Microcreadium*. It thus appears that the presence of setae on the tail, like many other characteristics of cercariae, is merely a larval adaptation with little phylogenetic significance. Palombi (1934*b*) has shown that one of the setiferous-tailed cercariae described by Monticelli develops into the adult trematode, *Lepocreadium album*. This parasite utilizes *Nassa mutabilis* and *Conus mediterraneus* as the first intermediate host, *Aplysea punctata, Tapes decussatus* and *T. aureus* as the second intermediate host, and *Blennius gattorugine* as the definitive host. All of these hosts differ from those in the life cycle of *L. setiferoides*. The adult *L. setiferoides* has not been previously described. It is most like *Lepocreadium ovalis* Manter, 1931, found in the intestine of the pinfish, *Lagodon rhomboides,* collected at Beaufort, N. C. The body of *L. setiferoides* is about half as large as that of *L. ovalis,* while the eggs are about the same size. Manter does not give the dimensions of the pharynx or a description of the excretory bladder, the latter organ being very conspicuous in *L. setiferoides*.

Lepocreadium setiferoides may be of some economic importance since it parasitizes a food fish. These parasites have been found in very small flounders and a young animal generally is more susceptible to the injurious effects of parasitism than an older animal. However, larger flounders, six to eight inches or more in length, rarely appear to be infected. This probably explains the fact that Linton did not find this species in his extensive study of fish parasites.

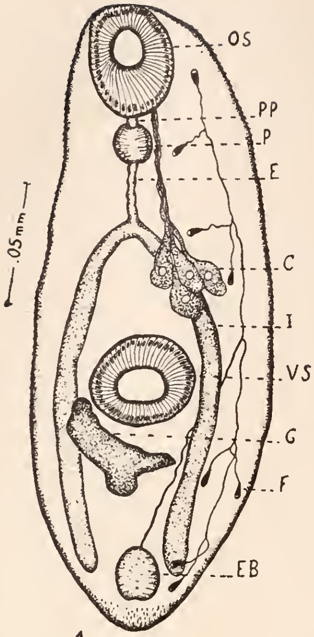
CERCARIA CUMINGIAE N. SP.

This trematode develops in sac-shaped sporocysts in the digestive gland of *Cumingia tellinoides*. Approximately 4 per cent of 175 *Cumingia* were found to be infected.

The Sporocyst (*Fig. 5*)

The sporocysts are simple, sac-shaped or tubular structures having at each end a concentration of cells that stain deeply with paracarmine. Otherwise, the walls of the sporocyst are very thin. Young sporocysts containing no germ balls or cercariae are approximately 0.20 mm. in length by 0.09 mm. in width. Older sporocysts containing 12 to 18 germ balls and cercariae are 0.58 mm. in length by 0.09 mm. in width, indicating that the growth of the sporocyst is principally in length.

PLATE II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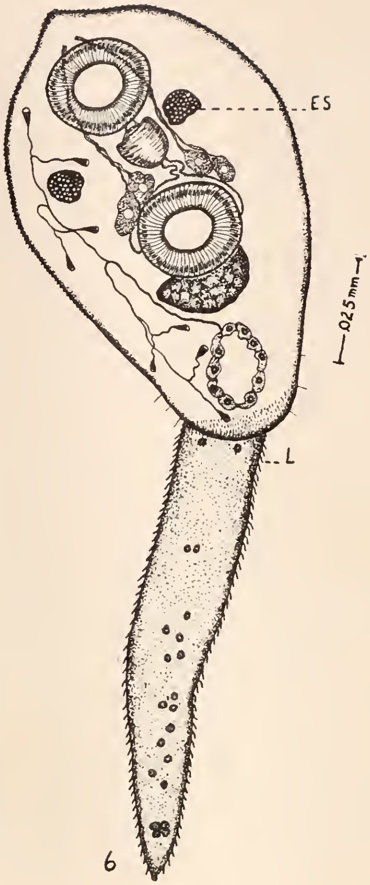

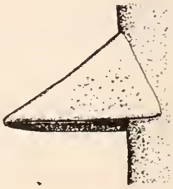

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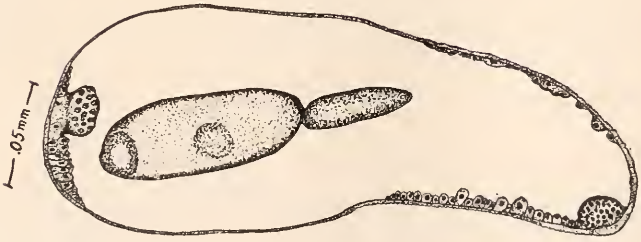

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Description of Plate II ?

All figures concern *Cercaria* ~~*tellinoides*~~ Cumingiae.
Fig. 4. Ventral view of metacercaria removed from cyst.
Fig. 5. Young sporocyst containing a cercaria.
Fig. 6. Ventral view of cercaria.
Fig. 7. Lappet from tail of cercaria (diagrammatic).

The Cercaria (*Fig. 6*)

Spination is limited to the cuticula of the anterior half of the body and a small area on the ventral surface near the posterior end. The body is oval in outline, varying in length and width with the degree of contraction. There are two conspicuous eye spots on the dorsal surface near the level of the pharynx. The oral sucker is subterminal and slightly smaller than the ventral sucker which is located in the mid-ventral region of the body. The ventral sucker bears about three rows of papillae. Immediately lateral and anterior to the ventral sucker there are four cephalic glands that stain deeply with neutral red. The ducts from these glands pass anteriorly to open externally on the anterior margin of the oral sucker.

The oral sucker is followed by a short prepharynx, a bulbous pharynx, a well-developed esophagus, and short intestinal crura which do not extend posterior to the ventral sucker. The short posterior extension of the intestinal branches is a rudimentary condition since they reach almost to the posterior end of the body in the metacercaria.

The excretory bladder is simple and sac-shaped and its wall is composed of a single layer of large granular cells. From the anterior portion of the bladder, the main collecting tubes pass laterally and anteriorly on each side of the body to the level of the ventral sucker where each divides into two secondary tubes, one of which extends anteriorly, the other posteriorly. Each secondary tube receives the capillaries of two pairs of flame cells so that the excretory formula equals $2[(2+2)+(2+2)]$.

The reproductive system is represented by an undifferentiated mass of cells located between the excretory bladder and the ventral sucker.

The tail of the cercaria is unusual in that it possesses, on each side, a row of cup-shaped lappets which are about 0.005 mm. long and can be raised to stand out at right angles to the tail or may be folded backward flat against the tail. There are fifty-two lappets on each side of the tail including the proximal first two which are not well developed. They may give the tail the appearance of being annulated.

Measurements in millimeters of cercariae killed and fixed under light cover-glass pressure are as follows: body length 0.1; width 0.06; oral sucker 0.026 by 0.027; ventral sucker 0.026 by 0.03; pharynx 0.01 long by 0.012 wide; eye spots 0.01 in diameter; tail, length 0.095 to 0.15, width 0.014 to 0.018. Measurements of living material give somewhat higher values.

In emerging from the host, *Cumingia tellinoides,* the cercariae are expelled through the excurrent siphon. They swim about in the sea water with the body folded upon itself and the tail describing figure-eight movements pulling the body along. The larvae show a definite negative response to light. When one side of the finger bowl receives more light than the other, the cercariae swim directly to the region of lesser illumination. They are frequently carried back into the clam by the sucking action of the incurrent siphon. Upon coming in contact with the tissue of the siphons or foot of the clam, they quickly lose their tails, penetrate the tissue and encyst. The siphons may become so filled with cysts that portions of them are detached. These infected pieces of siphons may retain their ability to move, simulating the motion of annelids, and thereby attracting the attention of fishes. This detachment of portions of the siphons seems to be partially autotomous and partially due to the rupturing of the siphon tissue by the metacercariae. Occasionally, *Cumingia* were found to be naturally infected with this metacercaria but with a fewer number of cysts than in experimental infections.

The Metacercaria (*Fig. 4*)

The young metacercaria is similar to the cercaria in most respects but certain changes occur with an increase in age. These changes include: the loss of the tail when penetration takes place; the disintegration of the eye spots; the elongation of the intestinal ceca so that they extend to near the posterior end of the body; the increase in the number of cephalic glands to four on each side of the body; and some differentiation of the genital ducts. The excretory system is the same as in the cercaria except that the excretory bladder is relatively smaller in the metacercaria. Measurements in millimeters of metacercariae removed from their cysts and flattened under light cover-glass pressure are as follows: average body length 0.195; average body width 0.088; oral sucker 0.031 by 0.032; ventral sucker 0.029 by 0.032; pharynx 0.018 by 0.021.

Specimens of *Cumingia* were experimentally infected with metacercariae and then segregated for varying periods of time up to two weeks to allow the metacercariae to mature. These infected *Cumingia* were fed to specimens of *Fundulus* and *Paralichthys*. Upon subsequent examination, no worms were found in the *Fundulus* but from the intestine of one flounder over fifty small worms were recovered and definitely found to have developed from the metacercariae that were experimentally fed. Control fishes were negative for this species. The worms recovered had not developed sufficiently from the metacercarial stage to determine, with accuracy, the characteristics of the adult.

DISCUSSION

Although *Fundulus* and *Paralichthys* did not serve as favorable definitive hosts, it is believed that some fish is involved in the life cycle of this trematode. This is suggested by the fact that *Cumingia* is a bottom-dwelling form, and since the siphons can be protruded some distance from the shell, they could be bitten off by fishes. In addition, the anatomical characteristics of this trematode suggest that it belongs to the family *Allocreadiidae,* the members of which, with few exceptions, are parasites of fishes.

The relationship of this species to other trematodes is uncertain since its life cycle is incompletely known. However, the arrangement of the principal excretory tubes, the sac-shaped excretory bladder, and the position of the genital primordium, suggest affinity to the family, *Allocreadiidae.* The cercaria has certain characters in common with *Cercaria myocerca* Villot, 1879, from *Scrobicularia tenuis.* Both of these larvae have the simple, sac-shaped type of excretory bladder. This seems rather significant since *C. myocerca* is a marine form with a setiferous tail and all other described species of setiferous-tailed marine cercariae have either a Y- or U-shaped, or a long tubular excretory bladder. Both species have eye spots. The molluscan host of *C. myocerca* is *Scrobicularia tenuis* which belongs to the same family as *Cumingia.*

SUMMARY

The life cycle of *Lepocreadium setiferoides* (Miller and Northup) has been experimentally traced. The rediae and cercariae develop in the snail, *Nassa obsoleta;* the cercariae encyst in annelids of the genus *Spio* and the turbellarian, *Procerodes warreni;* and the adult worm develops in the intestine of sand dabs and flounders. The structures of the various stages in the life cycle are described.

The anatomical features and most of the life cycle of *Cercaria cumingiae* n. sp. are described. The sporocysts and cercariae develop in the digestive gland of the bilvalve, *Cumingia tellinoides.* The cercariae encyst in the siphons, foot, and other organs of this mollusk. The adult worm probably develops in some fish. From the nature of the excretory bladder, the flame cell pattern, and the location of the genital primordium, it is believed that this trematode belongs to the family *Allocreadiidae.*

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AN EXPLORATORY INVESTIGATION OF THE SELECTIVE VALUE OF CERTAIN GENES AND THEIR COMBINATIONS IN DROSOPHILA[1]

JEAN BRIERLEY[2]

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The viability and vitality of fruit flies have interested geneticists ever since certain mutant stocks were found to be weaker in a number of ways than the wild stocks from which they had arisen. As Timoféeff-Ressovsky (1934) states, vitality of an organism is an indefinite quality to measure. It consists of a number of different factors such as duration of life, fertility, adaptability, mortality rate, resistance to disease, immunity, etc., only a few of which can be weighed and compared objectively. Fertility has been studied in several different ways. Saveliev (1928) and Timoféeff-Ressovsky (1933) have compared the total number of eggs or offspring; Lüers (1935) has studied deviations from the expected 1 : 1 sex ratio; Timoféeff-Ressovsky (1933 and 1934) and Csik (1935) have examined the alteration of 1 : 1 back cross ratios; and Timoféeff-Ressovsky has also considered the mortality occurring between the egg and adult stages in the life history of *Drosophila*. Pearl, Parker, and Gonzalez (1923), Gonzalez (1923), and Lüers (1935) have studied life duration as a criterion of viability by comparing life spans of males and females together or separately in different stocks. Comparing the life durations of males with that of females of the same stock has been done by Pearl and Parker (1924) and Lüers (1935).

Gonzalez (1923) found that different combinations of the second chromosome mutations black, purple, vestigial, arc, and speck had different effects on the longevity of the flies, and that one mutant gene added to a poor stock might improve it considerably. Other reports of similar circumstances are: an example of the combination of black and curved producing greater numbers of offspring than either stock alone, found by Bridges and Sturtevant (1914); a "white" mutant produced by heat which showed greater viability than the wild from which it arose, very briefly reported by Jollos (1932); and a gene for transparent eye which, when combined with it, improved a weakening effect produced by a gene for eye color in *Ephestia*, investigated by Kühn (1932) and Kühn and Henke (1929–1932). Other instances of genes influencing viability in different ways and to varying degrees have been worked out for several different gene combinations. Timoféeff-Ressovsky (1934) worked on *Drosophila funebris*, and with the genes eversae, singed, venae abnormes, miniature, lozenge, and bobbed. Csik (1935) showed the interactions of Lobe², Minute-173, and plexus to produce differences in fertility in *Drosophila melanogaster*. Gonzalez (1923), Csik (1935), and Lüers (1935) have shown quite conclusively that the factors which influence fertility and longevity (viability) of flies are the manifestations of the already known genes and their interactions, not unknown lethal genes.

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[1] Contribution from the Department of Zoölogy, University of Michigan.

Calculations for this paper were made with a calculating machine loaned to A. Franklin Shull by the Trustees of the Elizabeth Thompson Science Fund.

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In 1933 I became interested in the question of whether such occurrences were common, or at all general, and set out to test several combinations of genes in *Drosophila melanogaster*. Because I was chiefly interested in the way these combinations of genes might influence the survival value of the flies, the experiments were arranged to show not only comparative fecundity in different genotypes, but also comparative rapidity of production.

It is well understood that the rapidity with which offspring are produced is of great importance for the survival of the species. In this study, rapidity of production was measured in terms of the length of time between generations. This is the time that elapses between a parent's reaching the reproductive period and the mean time at which all of her offspring become mature, and is a function of the number of individuals in the family and the period in the lifetime of the parent at which they were produced. Selective value of a genotype consists not only in rapidity of production, but in the size of the family as well. Consequently, these two variants have been expressed together in the term Selective Index (S). S is used to represent the value in an evolutionary sense of flies of each genotype; and since this value varies directly as the number of offspring they produce, and inversely as the length of time between generations, it is the ratio of the former to the latter (mean number of offspring/mean time between generations).

I wish to express the deepest gratitude to Professor A. F. Shull not only for suggesting the problem but for furnishing invaluable help throughout its progress.

METHODS

Drosophila melanogaster was selected as the experimental animal, and in the three years during which the experiments were conducted, a total of nineteen different mutations were examined. Which of the hundreds of known mutations were to be studied was decided largely by chance and to some extent by the results of Gonzalez's work (1923). The nineteen mutant stocks used were: black, brown, claret, Curly, cut^6, Dichaete, eosin, eyeless, glass, hairy, miniature, purple, roughoid, scarlet, sooty, vermilion, vestigial, white, and yellow. Flies were kept in 70-cc. vials at constant temperature of 25° C. and fed on standard cornmeal-agar-molasses food medium.

Sixteen experiments were completed between November, 1933 and January, 1937. For all of these except the third, stocks were prepared from three mutant genes and their various combinations. Thus eight stocks were used, consisting of three single mutant stocks, three with two mutant genes, one with three mutations, and wild as a control. Experiment 3 differed in that six different mutant genes were combined with vestigial singly, so that the eight stocks consisted of the vestigial stock and six stocks with two mutant genes one of which was vestigial, plus wild. Experiment 1 and its repetition 1*a* combined the genes *y, w,* and *Cy*; Experiment 2 combined the genes *bw, D,* and *st*; Experiment 3, the genes, *y, w, v, st, ca,* and *ey* individually with *vg*; Experiment 4, *vg, bw, D*; Experiment 5, *y, pr, vg*; Experiment 6, w^e, *vg*, e^s; Experiment 7, *y*, ct^6, *gl*; Experiment 8, *m, b, gl*; Experiment 9, w^e, *vg, gl*; Experiments 10 and 10*a*, w^e, *pr, vg*; Experiment 11, ct^6, *b, st*; Experiments 12 and 12*a*, *m, pr, st*; and Experiment 13, *ru, h*, e^s. Experiments 1, 10, and 12 were repeated because of what was believed to be abnormality in one of the eight stocks.

In October, 1934, a process of "purification" was begun in several of the stocks which were to be used later in the experimental work. The method was patterned after one recorded by Timoféeff-Ressovsky (1933 and 1934), the purpose of which was to get all the mutant genes into as nearly as possible the same genetic environment, so that all flies would be essentially similar in all genes except the one for which the stock was named. This process was maintained continuously until the end of the experimental work, and those flies used in making up stocks for individual experiments were taken from these purifying stocks at various times. Of the nineteen stocks named, brown, claret, Curly, Dichaete, eyeless, vermilion, and white were not purified at all before using. Several of the stocks were not added to the purification process until the summer of 1935. Consequently flies in different experiments were purified for different lengths of time, varying from five generations to fifty-three generations.

The procedure in a single experiment was to use five vials for each stock, each containing one female and two males not more than twelve hours old. Single females were used rather than group matings, in order to show individual variations of the females. All forty vials were prepared and the flies added at the same time, in order to keep external modifying factors as nearly equal as possible. For the same reason the forty vials were kept in close proximity, and all flies were transferred at the same time. On the second day, fourth day, sixth day, etc., after the experiment was begun, all parent flies were transferred to new vials, until the females died. The original vials in an experiment were designated "*a*," the vials into which the flies were transferred the first time "*b*," the third vials in the series "*c*," and so on. If a male died he was replaced, so that there was the same number of flies in each vial at all times.

The technique used was not constant throughout the sixteen experiments. In the first eight experiments only one male was regularly used in each vial, but in Experiment 8 a second male was added to each vial in which no eggs had been produced by the eighth day. In all later experiments two males were kept in each vial, because it had been found that a female would sometimes mate with a second male when she had not successfully mated with the first one.

The first flies to emerge in a vial appeared sometimes on the eighth day after the parents were put into the vial, sometimes on the ninth, and often on the tenth day. All vials in one experiment were counted on the same day. Thus every vial was not counted on the first day that flies emerged therein. But after the first count flies were counted in each vial every forty-eight hours until five counts had been recorded. Some flies remained in the vials for two days (those that emerged later in the day on which the count was made), so that some of the population removed on the second day thereafter were old enough to have laid eggs. Consequently, there was the possibility of an F_2 generation emerging in the same vials any time after the tenth day.

It was determined by special treatment of the beginning of Experiment 9 that only 0.95 per cent of the flies emerging in the first four sets of vials were omitted by discontinuing counting after the fifth count. It is not likely that even this small percentage was omitted in all experiments or even later on in this particular one, because as parent flies grew older their offspring tended to emerge earlier, and also tended to be more concentrated in the first few counts, than earlier in the experiment.

RESULTS

The age of each female at death, the number of her offspring, and the length of time between her generation and that of her offspring was calculated, then a mean of each taken for each group of five females of the same genotype. For instance, the mean life span of the white-eyed females in the first experiment was 21.75 days, their mean production was 501.2 offspring, and the mean time between generations was 20.43 days. Those females that produced no offspring were included in the means although there is some possibility that they were not behaving as they would have done in a natural population. But since so little is known about the irregularities involved, and no special study of them was made in this research, it could not be assumed that they were abnormal enough to exclude them from comparison with others of their genotype.

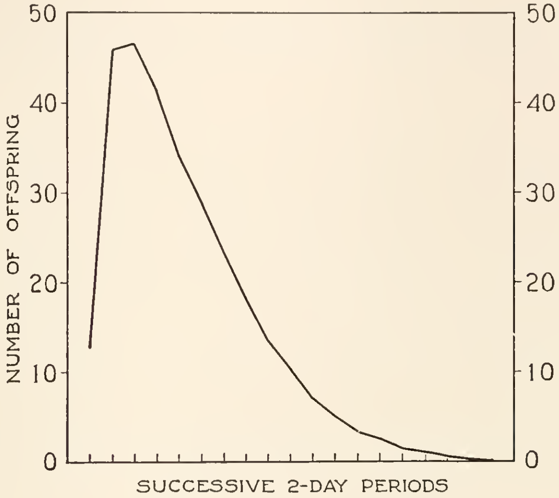

Fig. 1. Mean production curve of 593 females.

Because of accidental deaths and escapes the exact life span of some females was not known. In some instances in which a female's age could not be ascertained, her record up to the time of her escape showed that she would probably have produced no more offspring. There is a normal curve of production, which shows very clearly on the charts recording numbers of offspring in two-day periods. A rapid increase in the totals of the first few counts is followed by a gradual decline which may continue for many days, but gradually tapers off to zero. Figure 1 represents the mean of all recorded production curves. Several females laid eggs even after their production curve for offspring had dropped to zero, but these eggs did not hatch. In any case, if a female had not laid fertile eggs in three successive vials, her family was considered complete. It is well known that *Drosophila* females often live beyond their egg-laying period (Hanson and Ferris, 1929; Shapiro, 1932). Very few of the flies in these experiments laid eggs in the last several days of their lives, and many of them lived for many days after egg-laying had ceased. The families of escaped females, if the production curve showed that they were complete, were considered comparable to those more completely known, and were included in the means. If, however, a female escaped during her productive period, it was assumed that her family was still incomplete, and the number of her offspring up to the time of her escape was not included in the mean of that group.

Individual females showed wide differences among themselves both in length of life and in numbers of progeny. In any one experiment females supposedly identical genetically showed wide variations, and the same is true of females of the same genotype from different experiments.

A source of error in comparing the production of individual females may be in assuming that they all mated equally and that the mate of each was equally capable of fertilizing all of her eggs. Guyénot (1913) showed that when a female's supply of spermatozoa is exhausted, her egg-laying stops for a time and then may continue, but unless a new mating is accomplished these eggs are infertile. Castle, Carpenter, Clark, Mast, and Barrows (1906), Hyde (1914 and 1924), and Lutz (1914) also found that a female must mate more than once if all her eggs are to be fertile. The females in this investigation, at least those prior to Experiment 9, were under unnatural circumstances in so far as mating possibilities were concerned, because if their single mates were incapable of fertilizing all their eggs, their families would be smaller than they would have been if new matings could have been made later in life. There are only three females from these experiments which were obviously affected in this way, though there were probably others affected to some lesser degree. In the first experiment no males were replaced. Consequently the drop in a female's production after the death of her mate can be demonstrated. Table I presents the number of offspring produced in successive vials, by three females whose mates died early in their lives, compared with the mean number of offspring for the same period, from their sisters whose mates survived. The means were computed from all females of one genotype which had mates; consequently the three named females were included in the means until they were without mates. Means in whole numbers were considered accurate enough for the purpose of Table I. The heavy vertical lines dividing the three families represent the points at which the males died. After this time the female was alone in the successive vials. It will be seen that the number of her offspring in the two-day period directly following the death of her mate was for all three females normal. That is, the reduction in family size did not begin to manifest itself until the third day after the absence of the male. None of the females from which the means were drawn had as short productive periods as the three illustrated. One of the yellow white females ceased production in the " l " vial, and the other white and Curly females all continued producing at least through the " i " vials.

Many females went on producing offspring at the normal rate after their mates had died and had been replaced. Several females' production diminished in the counts immediately following such a change, then rose again to the level that was expected for their stage in the production curve. The explanation is probably that the female did not mate immediately with the new male. When a new male was added to a vial to replace a loss by death and the female's production decreased, just as happened in the three cultures illustrated, it could be surmised that they had not mated. In Experiment 3, one of the vestigial eyeless females illustrates a similar phenomenon. She laid no eggs during the first eight days of the experiment, but when a new male was added she produced 86 offspring within the next six days and continued thereafter in quite a normal way. In several vials in which the flies proved entirely sterile they were seen mating, so the fault was not always merely that they did not mate. These illustrations all contribute to the probability that the cultures were not all comparable in this regard, and that a number of the 99 females which were apparently sterile might have become productive if a suitable male had been present.

Table I

Vial of Series	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*	*m*	*n*
White ♀ 5	45	96	89	100	63	52	0	0	0	0	0	0	0	0
Mean	36	82	80	76	52	62	54	49	30	9	1	1	1	0
Curly ♀ 5	19	72	89	65	12	1	0	0	0	0	0	0	0	0
Mean	15	76	82	67	63	66	57	53	32	28	40	37	30	7
Yellow white ♀ 2	4	52	68	60	65	58	55	27	6	4	2	0	0	0
Mean	11	59	74	63	71	54	56	48	42	29	29	18	8	4

In determining the number of offspring produced by each female, no record was made of those that died in any of the immature stages of development. Those which did not live to reach the reproductive period could have no influence on the length of time between generations. Consequently, differences between the families of different females rest only on those offspring which reached adulthood.

Comparisons of Wild Females and Vestigial Females

The age records of the 75 wild females studied in this investigation varied from 3.25 to 57.75 days. The mean age at death of all 75 was 26.21 ± 1.42 days. Mean life spans of wild-type females of stocks used by other *Drosophila* workers have been reported as follows: Hyde (1913), 34.5 days (from a study of 97 females); Lutz (1914), 26.4 days (unmated females); Pearl and Parker (1922), 38.8 days ("expectation of life"); Gonzalez (1923), 40.62 ± 0.42 days; Pearl and Parker (1924), 48.0 days; Alpatov (1930), 49.11 ± 0.50 days; Gowen (1931), 33.1 ± 0.6 days; Lüers (1935), 20.1 ± 0.5 days. Pearl and Parker (1922) isolated several different "lines" of wild flies and found their longevity to differ considerably (from 22.04 ± 1.57 to 50.02 ± 0.85).

The ages of the 34 vestigial females varied from 47.75 days to 1.25 days, and the mean was 20.26 ± 1.63. Gonzalez (1923) found the mean of vestigial females from his stock to be 20.98 ± 0.4; Pearl and Parker (1924) found it to be 19.8 days; Hanson and Ferris (1929) found 20.13 days; Lüers (1935), 18.9 ± 0.2 days; Carver (1937), 28 days (average of 51 females). The numbers of females of other mutant stocks were not large enough to be employed in such comparisons; moreover, no other stocks have been measured as frequently by other workers as have these two.

Selective Index

The number of parent flies of each genotype in these experiments is too small to be used in computing reliable means either of fecundity or of longevity. Consequently, although the mean number of offspring is used in deriving the selective index, this figure must be considered as applying only to these particular flies and not to flies of these genotypes in general. The means used in calculating S for all the genotypes that were tested are those of fecundity and of time between generations, already mentioned.

The selective indices of all groups of flies which involved a particular gene and its combinations with other genes have been selected from the sixteen experiments and treated in such a way as to isolate the effect of that gene. To use the gene for black as an example, the S of the black flies was compared with the *S* of the wild control flies. When such a comparison was made in Experiment 8, *S* for black flies proved to be 2.01 times *S* for wild. These two stocks are supposedly identical except for the genes at the black locus. This comparison, then, should show whether the mutation from b^+ to *b* is of survival value to the flies. But in Experiment 11 the index for the same mutation (*b*) was only 0.14 times that for the wild flies. Such a discrepancy is probably caused by the individual differences of the twenty females involved.

Similarly the *S* of miniature black flies can be compared with the *S* of miniature flies, which are presumably identical except for the gene at the locus *b*. All such comparisons of flies carrying the gene *b* with flies identical except for this one gene were grouped together and the mean derived. The mean of all ratios comparing *b* flies with b^+ flies is taken as the value of *b* in comparing it to the other mean ratios of *S*'s, derived by a similar process, for each of the other genes used. Flies carrying the genes *v*, *ca*, and *ey* could be compared only once to flies with their wild-type alleles because they were used only in Experiment 3 which was carried out differently from the rest. Consequently their values, derived from ratios of *S*'s, are hardly comparable with those of the other sixteen genes.

Table II

Mean ratios of selective indices for all genes studied, arranged in order of magnitude

Gene	Mean Ratio of *S*'s	No. of Ratios of Which it is the Mean
Cy	2.57	8
w	2.14	9
h	1.80	4
w^e	1.76	16
pr	1.75	18
st	1.65	14
v	1.57	1
gl	1.41	11
vg	1.15	25
D	1.14	8
ey	1.09	1
ru	1.08	4
ca	1.04	1
y	0.95	17
bw	0.95	8
m	0.65	11
e^s	0.60	8
b	0.36	8
ct^6	0.25	8

In Table II are summarized all the mean ratios for *S* arranged in order of magnitude. *Ca*, *ey*, and *v* are included for the sake of completeness. Curly appears at the top of the scale, but it should be pointed out that it was tested in only two experiments (1 and its repetition 1*a*), and that in one of these the yellow flies were abnormally poor (compared to their performance in other experiments), making the ratio of *y Cy* to *y* flies very high. If this one ratio is omitted, and the mean derived without it, Curly drops to 1.29. White likewise is raised to its high rating because of the defective yellow flies in Experiment 1. Omitting that one ratio of 10.42, the mean ratio of white falls to 1.11. Hairy is even less secure than these two in its position, because its mean is drawn from only four ratios, all in Experiment 13. In the strictest sense, brown, claret, Curly, Dichaete, eyeless, vermilion, and white should not be compared on the same scale with the genotypes that had been purified. But eosin, purple, glass, miniature, sooty, and cut^6 at least, are comparable for the purpose of this table, and scarlet, vestigial, and yellow also, except for a few of their ratios which involve unpurified genes.

Gene Combinations

A few genes have been brought to light whose interactions have a decided effect on the survival value of their bearers. Scarlet and purple are the most striking example of this phenomenon. In Experiments 12 and 12*a* their effects, measured by their selective indices, are parallel:

Exp.	*pr*	*st*	*pr st*
12	15.34	7.80	39.42
12*a*	19.46	4.59	36.35

Thus the value of the two genes together seems to be far superior to either one alone. Other examples of the same type of interaction are: *y* and *Cy* in Experiment 1, *y* and *w* in Experiment 1, *vg* and *bw* in Experiment 4, *y* and *pr* in Experiment 5, *vg* and *gl* in Experiment 9, and *m* and *st* in Experiments 12 and 12*a*. It should be pointed out that in the repetition of Experiment 1 the interactions of *y, w,* and *Cy* are not at all parallel to those given for Experiment 1, in which it is believed that the yellow flies were defective.

The following are combinations in which a third gene, interacting with a combination of two, brought about a higher *S* than either genotype had before being combined: *y Cy* and *w* in Experiment 1*a*, *bw D* and *vg* in Experiment 4, w^e *vg* and *pr* in Experiment 10*a*, and *ru h* and e^s in Experiment 13. The examples from Experiment 1*a* and 10*a* are not substantiated in their parallel experiments, 1 and 10.

There are many more examples from these experiments in which a gene with low selective value is combined with one of higher value, the interaction producing a selective value intermediate between the two: *y* and *Cy* in Experiment 1*a*, *w* and *Cy* in Experiment 1*a*, *vg* and *D* in Experiment 4, *vg* and *pr* in Experiment 5, *vg* and w^e in Experiment 6, e^s and w^e in Experiment 6, *y* and *gl* in Experiment 7, *vg* and w^e in Experiment 9, *gl* and w^e in Experiment 9, *pr* and *vg* in Experiment 10, *pr* and w^e in Experiment 10*a*, ct^6 and *st* in Experiment 11, *b* and *st* in Experiment 11, *m* and *pr* in Experiment 12*a*, e^s and *h* in Experiment 13, and e^s and *ru* in Experiment 13. There are several examples of the latter type of reaction in which a third gene is added to a combination of two, the resulting flies being intermediate in selective value.

There are a great many examples of flies which had definitely lower value brought about by the interaction of genes. In this class belong not only flies carrying two mutant genes whose selective value was lower than flies carrying only one of the two, but those whose value was lower than a combination of two when a third gene was added. This type of interaction may be found in practically every experiment.

Combinations of scarlet and purple, brown and vestigial, and yellow and purple seem to be the most advantageous to flies in so far as their survival value is concerned. Scarlet and purple were combined with the same favorable results in two experiments, whereas the other two combinations were tested only once. In combinations of genes which result in flies intermediate in *S* between the values of the components, we may look upon one as an advantageous influence and the other disadvantageous. Thus eosin, purple, and scarlet, and less often glass and Curly seem to belong in the category "advantageous influence," and black, miniature, cut^6, sooty and less obviously yellow and vestigial seem to be most often "deteriorating influences."

DISCUSSION

Interaction of Genes

In this investigation there is some evidence that certain combinations of two genes produce flies of higher selective value than does either gene alone. If such indications can be seen from a study in which only nineteen genes were tested, it must be a rather general phenomenon, and a continuation of this sort of study should uncover more such circumstances. In this preliminary exploration of the field, at least three genes have been found that seem to carry their "good" influence into combinations with genes of weakening influence, and to bring about improvement in survival value of those flies. Purple in Experiment 5 and eosin in Experiment 6 are the most outstanding examples of this phenomenon. In Experiment 9 eosin, in combination with vestigial and glass, stands out as an advantageous influence again; and in Experiments 12 and 12*a* purple shows the same effect. But in Experiments 10 and 10*a* purple seems to be a weakening influence, and vestigial advantageous. Eosin in combination with vestigial, here, is very much lower than either alone. Whether this indicates that eosin is erratic in its "good" influence, or that something had happened to the purple stock, or whether all stocks were in a state of depression at the time these experiments were in progress, is not known, but it is true that two of the lowest values of S for the wild control stocks occurred in these two experiments (as shown in Fig. 3).

Glass, tested in three experiments, is sometimes in the rôle of "good" influence, sometimes not. Its advantageous tendency seems to be overbalanced by the damaging influence of the genes black, cut^6, and miniature. Scarlet likewise is not at all consistent, but does exert an advantageous influence on these three detrimental genes. Curly, although tested with only white and yellow, does seem to show promise of combining to good advantage.

It is interesting to note that these genes which have just been mentioned as the most advantageous influences discovered, at least in these experiments, are the same genes that appear in the majority of the upper ranks of Table II. Their high standing in that scale signifies that flies with these genes have a better chance of survival in an evolutionary sense, than do flies without these genes.

Vestigial, in all tests except those of Experiments 10 and 10*a*, appears to be a degrading influence, and almost every gene combined with it induced improvement in the combination. Black, cut^6, miniature, and sooty give promise of nothing but detrimental influence on other genes, and are practically lethal when combined. These are also the lowest four genes as listed in Table II.

Variability

Several factors dictate caution in the drawing of conclusions from the results of this research. The most outstanding hindrance is the variability of the flies. Females of the same genotype not only varied widely in their duration of life, but in their production, and in several factors connected with production.

(1) *Numbers of Offspring.*—Nonidez in 1920 wrote, "the rate of egg laying is variable in different females," and Shapiro (1932) ob-

served that "life span, egg laying period, and total egg output varied greatly from individual to individual in the various crosses." Although these were studies of egg production the same variation exists when reckoned in adult offspring. For the 647 females studied in this research, environmental conditions such as temperature, moisture, age of food, amount and consistency of food, light, ventilation, density of population (except for the difference of one male in about half the vials), etc., were as uniform as it was possible to make them, yet in only seven instances did two females of the same genotype in any one experiment have equal life spans and at the same time produce approximately equal numbers of offspring. Among 34 other cases in which two (or three) females of the same genotype lived equally long (excluding pairs of which one member was sterile), differences in production were considerable. The difference in output between the two individuals of any of these pairs varied from less than 100 to nearly 700, and the difference was as variable and as great among the shorter-lived pairs as among the longer-lived ones.

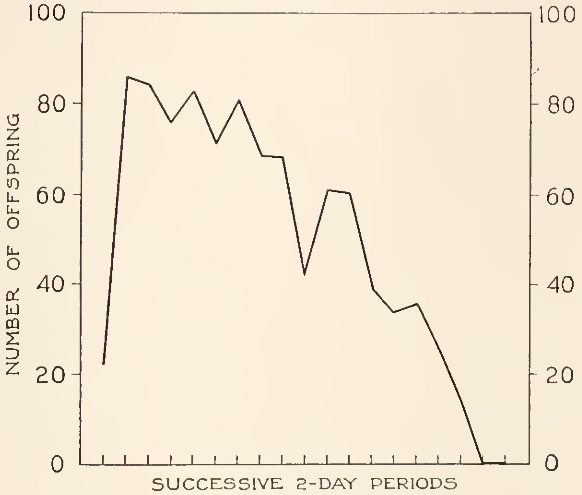

Fig. 2. Offspring production curve of a wild female from Experiment 4.

Individual females produced widely different numbers of offspring in successive periods of equal length. This form of variation has been referred to by other *Drosophila* workers in the past. Hyde (1913) reported cyclic production of individual females. Hanson and Ferris (1929) had evidence of somewhat the same phenomenon in six of their females. Their graphs of daily egg production show the same irregularities of outline from day to day that my graphs show for production of imagos. A curve such as that in Fig. 1, drawn for any one female, shows great irregularities in height for successive two-day periods (see Fig. 2). Dobzhansky (1935) wrote that "the number of eggs deposited in the same culture on successive days is notoriously variable," but his study was of groups of flies, not individual females.

(2) *Variation of a Single Stock in Several Experiments.*—These individual variations are probably the chief reason for the difference found between groups of five females of the same genotype in different experiments. A graphic demonstration of this is the comparison of *S* of wild control stocks in all sixteen experiments. In Fig. 3 the *S*'s are plotted as ordinates and the experiments arranged in their chronological order from left to right, spaced in their proper seasons. This arrangement has been used in order to show that in so far as can be judged from a small number of experiments, seasonal variations were not the cause of the fluctuations in *S*. It can be seen that the highest *S* of all occurred in Experiment 4 which was conducted in midsummer, yet three of the lowest values of *S* are from Experiments 10, 10*a*, and 11 which were also summer ones. Likewise both high and low *S*'s occurred in fall and winter seasons.

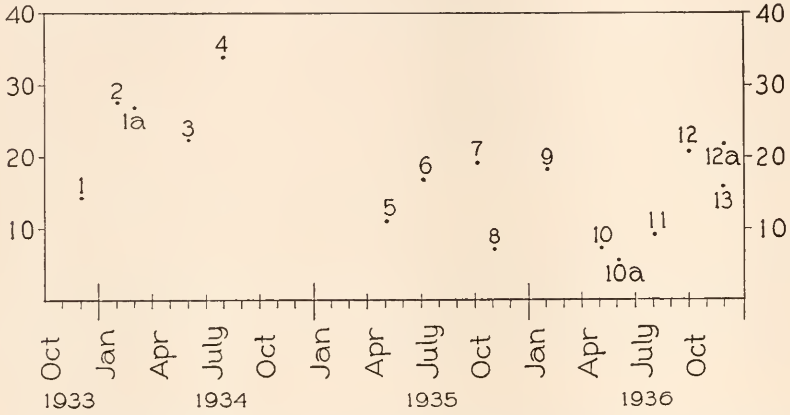

FIG. 3. Selective index of the wild flies in each of the sixteen experiments spaced chronologically.

(3) *Time of Development.*—Another form of variability occurred in the time of development of the flies. The offspring counted in these experiments developed from eggs that were laid in 48-hour periods, yet the imagos emerged over a period varying from eight days after egg-laying began in the vial, to as much as twenty-four days. The latter time interval between egg-laying and emerging of the adult could only

be measured in the specially treated first part of Experiment 9, where the late emerging offspring were saved and recorded. In all other experiments it could only be certain that offspring were emerging over a period as long as ten days.

(4) *Vigor.*—Variations in life span were often dependent upon the vigor of the flies. Those that were weaker and less active became more often caught in the food, and died. The more active ones spent more time on the stopper and dry sides of the vial and hence had less opportunity of sinking into the sticky food.

Inequality of Stocks in Amount of Purification.—It was not feasible to purify all stocks equally before using them in the experiments. Since no stocks had been purified before this study was undertaken, it would have entailed too long a delay before the work could be launched, if it had been decided to wait until all stocks were sufficiently pure. All through the experimental work tests were being performed to see whether certain genes could be used together, and until it was known whether a stock could be used, in most cases, it was not purified. The purification process itself involved so much labor that no more stocks could be maintained than were necessary. Consequently some of the stocks, those in the first several experiments, were not purified at all, and others for different lengths of time. This inequality of stocks detracts somewhat from their value in comparisons.

Comparative Reliability of the Several Factors

Since selective index is theoretically the correct measure of the success of one genotype in comparison to others in an evolutionary sense, it is well to consider whether fecundity, length of time between generations, or length of life might be substituted for S without serious reduction in reliability of the tests. Since S takes into account not only the number of offspring produced, but also the rapidity with which they are produced, it is logically superior to any form of evaluation which embraces only one of these qualities. Life duration as a measure of advantage or survival value has no correlation with S. This is to be expected since length of life has no necessary bearing either on the number of offspring or on their rapidity of production. There were several females in these experiments which lived longer than average, yet produced few or no offspring.

Time between generations, although the values for it are statistically the most reliable of any of the four criteria, is not by itself a valid measure of survival value because it often happens that a group of flies which has a high mean number of offspring will also have a relatively long mean time between generations. This large total of progeny is apt to be distributed over a longer period than is true of another group which has small productive capacity. That is, the general shape of the production curve for the two groups is the same at the beginning (the rapid rise in the first few periods), but for the low fertility group the height of the curve usually decreases soon thereafter, gradually and irregularly, while the curve of the high fertility group not only goes higher usually, in the first few counts, but stays high for a longer time before declining, or it may decline then rise again to a lesser peak. The decline is usually more gradual than in the lower fertility group, thus making the mean time of production longer. Thus a genotype which had the lowest mean time between generations in one experiment often was the least productive stock, and although all of the offspring were produced early, there may have been more offspring of another stock at that same time, even though the mean time between generations of that other population is longer.

The selective index, which has been introduced as a means of measuring the value of genes to flies in their survival in an evolutionary sense, is more reliable in evaluating the success of different types in survival than any of the heretofore measured viability factors. It is a numerical value and in order to use it for comparisons between genotypes, large numbers of flies must be raised and tabulated, and there remains a great deal to be done before wholly reliable conclusions regarding the comparative selective values of any of the *Drosophila* genes can be estimated. I consider the evidence that this investigation offers as being at least indicative of what sorts of things may be found when more reliable bases of comparison are obtained.

SUMMARY

1. A total of 203,159 flies (offspring) were counted in this investigation. Of this number, 190,318 were the basis of the selective index calculations. (The remaining 12,841 offspring were in families which were not comparable with the others for several reasons.)

2. In sixteen experiments a total of 647 females were compared, in groups of about forty, in respect to longevity, fecundity, and rapidity of production.

3. A wild-type female in Experiment 7 had the longest life span of all (57.75 days), and the longest mean duration of life of any one group was exhibited by the five wild females of that same experiment (42.25 days). Other groups which ranked comparatively high in longevity were the yellow cut^6 females of Experiment 7, the eosin ones of Experiment 6, and the yellow white ones of Experiment 1*a*.

4. The highest fertility record (taking into account only adult offspring) was exhibited by one of the eosin females in Experiment 6 (1434 offspring). The highest mean number of offspring of any group was found in the group of which this female was a member, the five eosin females of Experiment 6 (1046 offspring). The yellow purple females of Experiment 5 also showed high fecundity.

5. Selective index, the value of an individual in selection, is calculated by dividing the mean number of offspring by the mean number of days between generations. This is logically a better criterion of value to a race than is either longevity or fecundity.

6. Nineteen genes and their combinations, mostly in groups of three, were compared in respect to selective index.

7. In general, the addition of a mutant gene to another mutant gene or to a combination of mutant genes may (1) raise the selective value of the final combination above the value of either of the component mutations or combinations taken singly; (2) result in a selective value intermediate between those of the component mutations or combinations; (3) yield a selective value lower than the lowest of the components; or (4) make the selective value practically equal to that of one of the components. Some of the more striking examples of these reactions are mentioned below.

8. Flies which carried eosin, in combination with practically any other gene tested, had greater survival value than flies with the same genetic composition except for eosin.

9. Purple and scarlet genes are likewise advantageous, but less consistently so than eosin.

10. The genes cut^6, black, miniature, sooty, and vestigial have been demonstrated to produce a weakening effect when combined with several other genes separately.

11. It has been shown that females go on producing offspring at a normal rate for at least two days after the removal of their mates. Thereafter the number of offspring falls off rapidly.

12. Variability of females of the same genotype in production, longevity, vitality, and the time of development of their offspring renders uncertain the application of the conclusions of this investigation to *Drosophila* females in general.

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REACTIONS OF MARINE FISHES TO TEMPERATURE GRADIENTS[1]

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INTRODUCTION

The ability of fishes to respond by movement to experimental gradients of various physical and chemical characteristics of water and so to exercise selection of their environment has been repeatedly demonstrated.[2]

Studies of this type have thrown much light upon the senses and the behavior of animals. Furthermore, many workers have held that selection in an experimental gradient is indicative of the nature of the most favorable environmental conditions for the organism in question, even when the detrimental effects of conditions which are rejected can not be demonstrated. The term "optimum" has frequently been used in this connection (M. M. Wells, 1914, 1915 *a, b;* Mendelssohn, 1895; Herter, 1924, etc.). Conclusions as to the relative importance of different characteristics of water as indices of the suitability of fish environments have been drawn from the relative vigor of the reactions of fishes to these stimuli (Shelford and Allee, 1913). Speculative attempts to explain the curious migration of fishes, such as those of the anadromous salmonids, in terms of reactions to physical and chemical gradients, have been made by most workers in this field.

One objection to the view that an optimum is selected, namely, that animals can become acclimatized to conditions which are rejected, and that such acclimatization may alter their reactions to a gradient, has been pointed out (Jewell and Brown, 1924; Brown and Jewell, 1926). Shelford (1929) recognized this objection and concluded that the gradient method is more useful as a measure of "acclimation," rather than of habitat preference. Owing to the lack of sufficient data relative to the influence of acclimatization, its importance could not be estimated. The present investigation was undertaken to obtain more complete data on the basis of which the physiological and ecological significance of selection in an experimental gradient could be evaluated.

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[1] Contributions from the Scripps Institution of Oceanography, New Series, No. 42.

[2] For review of early literature see Chidester (1924).

Temperature has been regarded as having an important influence upon the migrations and distribution of fishes (Chidester, 1924; Ward, 1921; Creaser, 1930). Wells (1914), Shelford and Powers (1915), and Chidester (1922) found that fishes are very sensitive [3] and will react to this stimulus, but these reactions have not been adequately investigated. For these reasons most of the present experiments were performed with temperature (and a few with salinity) gradients.

I am indebted to Dr. F. B. Sumner and other members of the staff of the Scripps Institution for valuable advice and assistance.

Material and Methods

The material used was chiefly the young of the greenfish, *Girella nigricans* (Ayres). They were taken in rock-pools in the intertidal area near the Scripps Institution. Pacific killifish (*Fundulus parvipinnis* Girard) from False Bay near San Diego, young atherinids,[4] taken in the same locality as the *Girella,* and a few other species were also used.

The fishes were kept in the laboratory in flowing sea water, which was nearly at the same temperature as the surface water in which *Girella* were living in nature. In acclimatization experiments they were kept in constant temperature tanks, in which flowing sea water was maintained at any desired temperature by thermostatic control.

A satisfactory horizontal temperature or salinity gradient can not be maintained in a simple gradient tank of the type generally used. Owing to differences in density between the inflowing streams of water, stratification results in a steep vertical gradient. The tank which was devised for this purpose (Fig. 1) was a wooden one 80 inches long, 8 inches wide and 9 inches deep. It was subdivided by vertical partitions (*a*) into ten octagonal compartments arranged in linear series and communicating at their adjacent sides. Other vertical partitions (*b* and *e*) at the top and bottom further reduced the size of the openings (*p*) between adjacent compartments to two inches high by three inches wide. A horizontal wire screen (*s*) was placed over the lower partitions (*e*). The fishes were confined above this screen and, because of the shape of the compartments, could find their way from one compartment to the next without any difficulty.

Water in very small streams was introduced into and drawn out of each compartment at an equal rate through horizontal tubes (*i.t.*

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[3] Excellent work of another type on the temperature sensitivity of fishes has been done by Bull (1928, 1936).

[4] These were either *Atherinops affinis* (Ayres) or *Atherinopsis californiensis* Girard. The specimens were lost before they had been identified.

and *o.t.*) with several perforations, so that no horizontal currents passing from one compartment to another were present. Sea water heated or cooled to a high, a low, and an intermediate temperature first flowed through three large tanks in which it was vigorously aerated with compressed air. From the aeration tanks the water was distributed to the

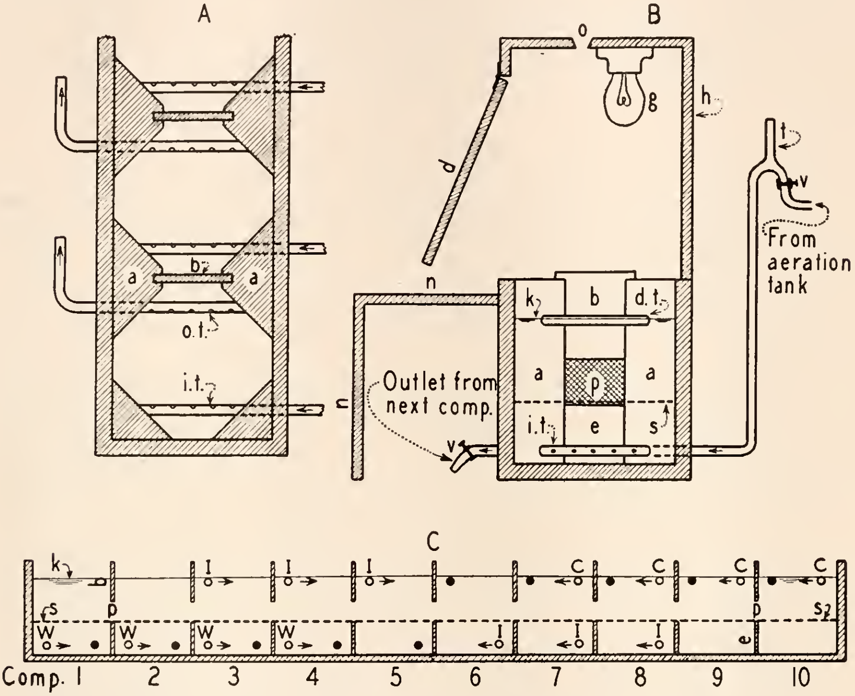

FIG. 1. Diagram of the gradient tank. *A*—Top view of a portion of the tank (Compartments 9 and 10). *B*—Cross-section through the tank (Compartment 2) and hood. *C*—Longitudinal section through the entire tank, showing arrangement of inlets and outlets. Cross-sections of outlet tubes are indicated by solid circles and inlet tubes by open circles. The relative temperature of the inflowing water is indicated above each inlet tube: *W* = warm, *I* = intermediate, *C* = cold. Dummy tubes are not indicated.

a, b, and *e* are partitions; *p*—opening between compartments; *s*—horizontal screen; *h*—hood; *d*—door of hood, *o*—observation slit, *g*—lamp; *i.t.*—inlet tubes; *o.t.*—outlet tubes, *d.t.*—dummy tube, *t*—air trap, *v*—screw-clamp, *n*—hood over outlets, *k*—water level.

gradient tank through 14 tubes, each of which was provided with a trap (*t*) to remove air bubbles and a valve (*v*) for regulating the flow. In every case water at a higher temperature than that maintained in any given compartment was introduced into it at the bottom (below the screen), while colder water was introduced at the surface. Dummy inlet and outlet tubes (*d.t.*) were provided to make all compartments similar.

In this tank a very steep and uniform horizontal gradient could be maintained without the presence of vertical gradients. A difference of 18° was usually maintained between the two ends, or 2° between adjacent compartments.

A hood (*h*) was attached over the tank. The tank was illuminated by five small electric lights (*g*) attached to the top of the hood. The movements of the fish were observed through a narrow slit (*o*) in the top of the hood. The room was darkened while observations were made, and the observer was apparently invisible to the fish.

Observations were made in the following manner. One specimen at a time was put into the tank at any desired temperature (usually the temperature from which it had been taken), the fish being screened off from adjacent compartments. After a minute it was released and allowed to swim freely about the tank. The door of the hood (*d*) was then shut and, after the fish had been in the tank for a given length of time (usually one hour), the observation was begun. A bell metronome was then started and the position of the fish recorded at six-second intervals. The observation was generally continued for 30 minutes (300 recordings). The approximate percentage of the time that the fish had spent in each compartment was then calculated, and the temperatures in them recorded. About 600 such observations were made in the course of these experiments.

Results

Experiments with Girella Kept at Current Seasonal Temperatures (14.5° to 22°) and Under Relatively Normal Conditions of Illumination

Specimens kept in the laboratory under these conditions for many months were found not to differ in their behavior and reactions from those recently taken.

In control experiments, in which the water flowing into the gradient tank was at the same temperature throughout and, consequently, no gradient was present, no consistent preference was shown for either half of the tank nor for any compartment except the ones at the ends of the tank (Fig. 2, *C*). When, however, a suitable temperature gradient was established, the fishes showed a marked avoidance of the two ends of the tank. After a short time an apparent "preference" for one or two adjacent intermediate compartments was established, all others being avoided. When the percentage of time spent in each compartment by a single individual in the course of a 30-minute observation was plotted against the temperature, a fairly regular, although frequently skewed, unimodal frequency distribution was obtained. The temperature at the mode of this distribution, located by a uniform procedure, will be referred to as the selected temperature.

While the temperatures selected in different observations by fishes taken from current seasonal temperatures varied considerably, a tendency to select high temperatures in the vicinity of 26° was observed.

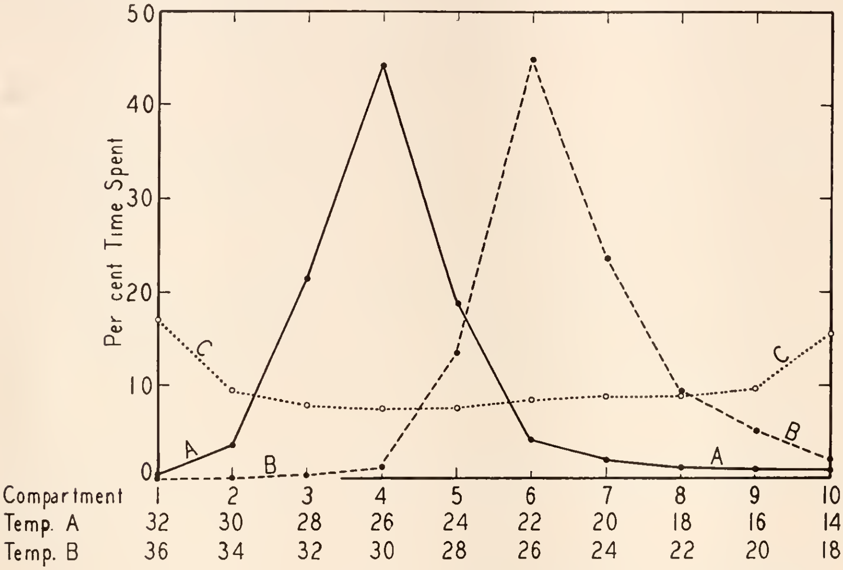

FIG. 2. Distribution of time spent in different compartments by *Girella* taken from sea water at the normal seasonal temperature and tested in *A*—a 32° to 14° gradient, *B*—a 36° to 18° gradient, and *C*—in the absence of a gradient (typical control experiment). Distributions *A* and *B* are based on the same eight specimens. The temperatures maintained in each compartment in the two experiments are indicated. Distribution *C* is based on five specimens. Observations made one hour after introduction of the fish.

So long as the two ends of the gradient were cold and warm enough to be invariably strongly repellent, the mean selected temperature was found not to be affected by changes in the range of the gradient used. This result is illustrated in Fig. 2. It will be observed that the modes of both distributions A and B are at 26°. In a number of such experiments the same result was always obtained. This shows that the fish were attracted to the selected compartments by the temperatures present there, rather than by any other differences (such as convection currents, etc.).

Figure 3 shows the distribution of selected temperatures obtained in 100 experiments in various gradients (usually 14°–32° or 15°–33°). The mean (25.7 ± 0.1°) is somewhat lower than the modal temperature (26.4°). Experiments were performed to determine the possible influence of size, recency of feeding, and season, which could account for the variability and the skewness of the distribution, since such skewness would result from unequal representation of specimens so differing. The mean temperatures selected by three size-groups of 14 specimens each (with mean weights of 9.6, 3.8, and 1.7 grams), taken at the same time, did not differ significantly. Similarly, groups of fishes which had been recently fed, starved for two to three days, and starved for two weeks selected, on the average, practically identical temperatures. Specimens tested in early fall and in early spring did not differ appreciably. The former had been living through the summer at 19° to 22° and, when tested, were taken from 20°. The mean temperature selected by 24 specimens in a 14°–32° gradient was 26.0°. The latter had been living through the winter at 14°–16° and were taken from 15–16°. Their mean selected temperature in the same gradient was 25.8° (22 observations). This indicates that, over a wide range of

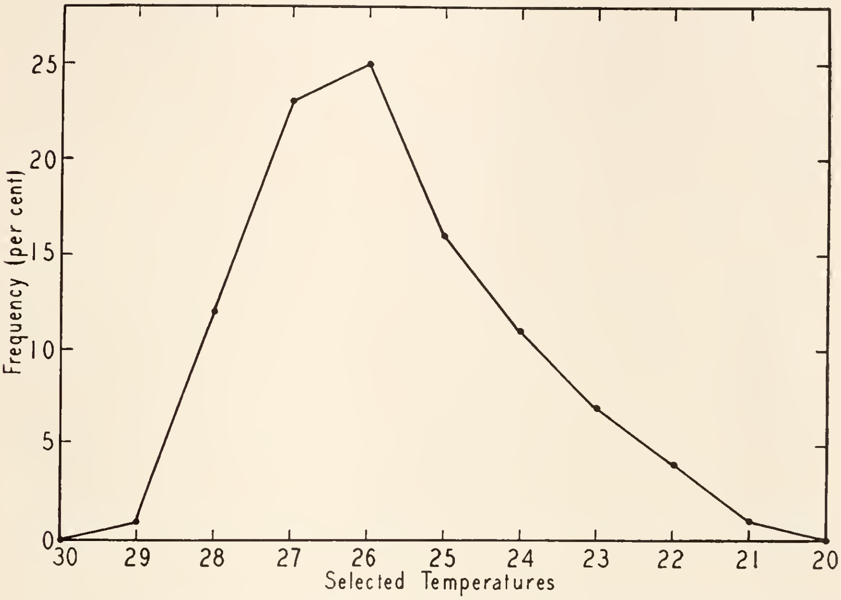

Fig. 3. Distribution of temperatures selected by *Girella* taken from current seasonal temperatures. (Based upon 100 thirty-minute observations, made one hour after introduction of fish into the gradient tank.)

temperatures, previous temperature history has no influence upon selection. The temperature of the compartment into which the fishes were introduced was found to have no influence upon the temperatures which were finally selected.

Since the temperature gradient was, of necessity, accompanied by other gradients, the possibility that the fishes had been reacting to some factor other than temperature was investigated. No gradient of oxygen or nitrogen tension was present, the water being brought to the saturation point at all temperatures by "aeration," as described earlier.[5] On the other hand, a gradient of the *concentration* of these gases was necessarily present. However, when the "aeration" of the water was reduced, with the result that the oxygen concentration at the extreme warm end of the gradient (32°) was equal to or higher than that present at 26° in the fully "aerated" gradient (4.75 cc./1.), the fishes continued to reject the high temperatures. Obviously, it was not to this gradient that they had been reacting.

The carbon dioxide tension could not be equalized by the amount of "aeration" provided. Consequently the CO_2 tension and the hydrogen ion concentration were slightly higher at the warm end than at the cold. Since Powers (1921) and others have found that some fishes will react to small differences of pH or CO_2 tension, experiments were performed to test the possibility that the fishes were reacting to these gradients instead of to temperatures. A shorter gradient (four compartments), in which the fish was given a choice between two contrasting temperatures, was used for this purpose. Water at an intermediate temperature was aerated and then passed at a constant rate through a tall cylinder, where CO_2 gas was bubbled through it. It then passed through a chamber, to allow for thorough mixing with the gas, and was then distributed to two lead coils immersed in flowing cold and warm water. The cooled and heated streams of CO_2-treated water were distributed, out of contact with air, to the cooler and warmer halves, respectively, of the gradient tank. No difference of pH was detected when samples of water from the two ends of the gradient were brought to a common temperature, showing that no difference of CO_2 content had been present. In a preliminary experiment, in which the water was not treated and a gradient of 26° to 32° was maintained, ten specimens invariably selected the cooler half of the gradient, spending 92 per cent of the time there. The gradient was then changed so that the warm end was at 26° and the cold at 18°. The water was treated with enough CO_2 to raise the partial pressure of CO_2 and the H-ion concentration at the two ends (26° and 18°) to higher values than those which had been present in the preliminary experiment at the corresponding ends of the gradient (32° and 26° respectively). Only the pH at room temperature (22°) was determined. The charge of pH necessary to satisfy the above requirements was readily calculated by the use of tables giving the relation between temperature, pH, partial pressure of CO_2, and total CO_2 in sea water of constant chlorinity and titratable alkalinity furnished by Buch et al. (1932). The pH of the water (chlorinity $18.6^0/_{00}$) was lowered from 8.1 to 7.7, and later to 7.35. In both cases the change was more than sufficient to satisfy the requirements. Twelve specimens tested in the treated gradients invariably selected the warmer half (26°), spending 91.1 per cent of the time there. Had the fishes been reacting to CO_2 tension or to pH instead of temperature in the untreated gradients, they should now have selected the colder half (18°), as they did in the untreated 26°–32° gradient.

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[5] Frequent oxygen determinations were compared with solubility values given by C. J. J. Fox (1909), corrected for vapor pressure. The untreated sea water was at an intermediate temperature and approximately saturated with air. The warmed sea water was, consequently, super-saturated with air, and "aeration," in this case, resulted in the removal of excess air.

While *Girella* is quite resistant to low salinities, it is a typical marine fish and does not occur in situations where the water becomes appreciably diluted. Experiments were performed to compare its reactions to temperature and to salinity, in order to test the value of these reactions as an index of "habitat preference." In a salinity gradient, in which the temperature was the same throughout and the salinities ranged from $5.6^0/_{00}$ to $29.9^0/_{00}$, no consistent reactions were observed. A combined temperature and salinity gradient was then established, with salinities ranging from $2.6^0/_{00}$ to $32.3^0/_{00}$ and temperatures from 26° to 18°, the dilute water being at the higher temperature. Seven specimens taken from normal sea water (salinity $33.8^0/_{00}$, temperature 18° C.) invariably avoided the normal sea water and spent 94% of the time in the five compartments in which the water was warmer and more dilute. The mean temperature and salinity which were selected were 24.6° and $7.8^0/_{00}$ respectively. The salinity gradients were not entirely satisfactory, since the fresh tap-water used differed from the sea water in several other respects. The pH was approximately the same, but the CO_2 tension was slightly higher, and a small quantity of free chlorine (.02–.03 parts per million) was present. However, this circumstance only emphasizes the fact that the fishes were selecting unnatural conditions.

Experiments with Girella from Constant-temperature Tanks

Three constant-temperature tanks, maintained at 10°, 20° and 30°, were used. Figure 4 shows the relation between the temperatures of acclimatization and the temperatures selected by specimens kept at the former temperatures for 3 to 4 months. It will be noted that the fishes from the 20° constant-temperature tank selected lower temperatures, on the average, than those from untreated sea water (discussed in the preceding section) taken from the same temperature in fall or taken from a lower temperature (15°–16°) and tested at the same time as the 20°-conditioned ones. Apparently, the small difference between the fishes from normal seasonal temperatures and those from the 20° constant-temperature tank (2.5° ± 0.23°) was due neither to season nor to the temperature of acclimatization. It was probably due to a slight difference of behavior.[6] The fishes in the constant-temperature tanks had been subject to conditions of dim and erratic illumination, and had been frequently disturbed. Some irrelevant factor of this nature was probably responsible for the slight change.

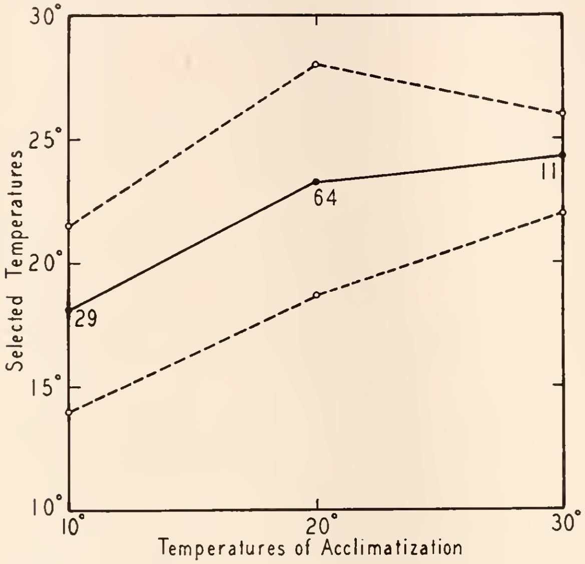

Fig. 4. Relation between temperatures of acclimatization and temperatures selected by *Girella* which had been kept for 3 to 4 months in three constant-temperature tanks. Solid circles, connected by solid lines, indicate mean selected temperatures. The number of observations in each group is indicated. Open circles, connected by broken lines, indicate the highest and lowest temperatures selected by specimens from each of these tanks. Observations made one hour after introduction of fish into gradient.

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[6] The fishes from the constant temperature tanks were more active, in the sense that they moved about the gradient more freely and reacted less sharply and rapidly. The distribution of selected temperatures was symmetrical.

At any rate, the specimens in the three constant-temperature tanks had been subject to the same conditions with the exception of temperature and are, therefore, comparable. The mean temperatures selected by eleven 30°-conditioned and eleven 20°-conditioned fishes, acclimatized to these temperatures for three months and tested in alternation in a 15°–33° gradient, were 24.3° and 23.6° respectively. The difference was small and the probability that it was significant was not high (.8578).[7] The 10°-conditioned fishes, however, selected considerably lower temperatures. When sixteen of these and an equal number of 20°-conditioned specimens were tested in a 12°–30° gradient, the mean selected temperatures were 17.8° and 23.2° respectively. The difference was unquestionably significant, the probability being .9999+.[7] The 20°-conditioned fishes were then transferred to 10° and the 10°-conditioned ones to 20°. They were tested again after they had been at the new temperatures for three days and again after 24 days. Other specimens, which had been kept continuously at 10° and 20°, were tested at the same time as controls. The result is shown in Fig. 5.

Results essentially the same as the above were obtained in other experiments, in which different periods of acclimatization and various gradients were used. When 10° and 20°-conditioned specimens were left in the gradient tank and repeated observations were made in the course of 24 hours, no appreciable changes of the mean selected temperature were observed after the first half hour, although the temperatures selected by single individuals in successive observations varied considerably.

General Notes on the Behavior of Girella

After the fishes had been introduced into the gradient, some were very active and showed little selection until they began to come to rest. As a general rule, however, a specimen taken from a relatively low temperature avoided, at first, the high temperatures which were later selected. As it moved back and forth in the gradient, its excursions into warmer water became more prolonged and extended, while those into colder water became more brief. The selected temperature thus moved up rapidly, until the fish came more or less to rest at a relatively high temperature. Thereafter, the selected temperature did not remain constant, but occasionally fluctuated up and down, so that the temperature which was selected at one time was avoided at another. The fish continued to make frequent excursions into other temperatures. A rapid excursion into a much higher or lower temperature was followed by a very rapid and violent reaction. But, if the fish moved into the same temperature gradually, the avoidance reaction was often markedly delayed and the excursion frequently followed by a temporary or a relatively permanent rise or fall, respectively, of the selected temperature. When the same specimens were tested more than once, no marked tendency was observed for the same individuals to select relatively high or low temperatures in successive trials.

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[7] For method of computing probabilities see Tippett, 1931, pp. 80–82.

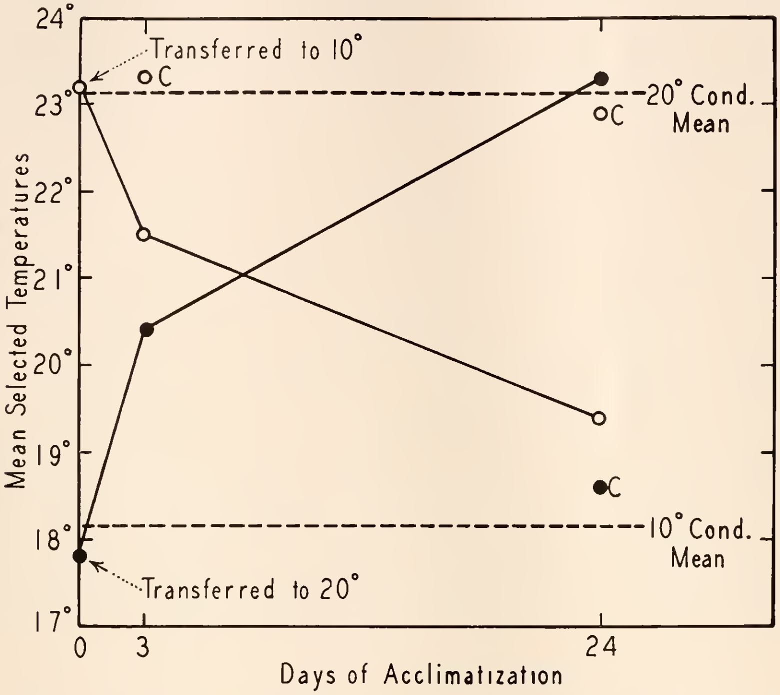

Fig. 5. Rate of change of mean temperatures selected by *Girella* as a result of acclimatization to 10° and 20°. Fishes originally conditioned for 100 days to 20° (open circles) and to 10° (solid circles). Circles connected by solid lines are mean temperatures selected in successive observations by 16 specimens of each group before and after transfer to the other temperature (as indicated). Days of acclimatization (abscissas) refer to time at new temperatures only. Points marked *C* are controls and are based upon 13 to 16 specimens which had been kept continuously at the original temperatures of acclimatization. These were obtained at the same time as those for recently transferred specimens on the same abscissa. Broken lines indicate mean temperatures selected by all the specimens which had been kept at 10° and 20° continuously for 100 days or more.

From these observations it appears that the variability of temperatures selected by different individuals with a similar history was not indicative of any permanent physiological differences, resulting in differences of "preference." A specimen could become "adapted" to, and establish an apparent "preference" for any temperature within a fairly wide range. Thereafter, other temperatures were repellent by contrast. Over a wide range of temperatures, however, the influence of past experience was, apparently, of brief duration and past impressions were rapidly destroyed in the course of the random movements of the fish in the gradient. Consequently, the range of temperatures at which the fishes finally came to rest was largely independent of previous temperature history. Specimens conditioned to 10° were unable to tolerate rapid transfer to the highest temperatures selected by specimens which had not been subjected to this abnormal temperature. The observed change of behavior was obviously adaptive.

Experiments with Other Species

In control experiments, in which no gradient was present, young atherinids behaved much like *Girella,* showing no "preference" except for the end compartments. In a temperature gradient their behavior was not quite as satisfactory as that of *Girella,* owing to a more pronounced tendency to move about in set patterns, rather than at random. Nevertheless, marked reactions to the gradient were observed. Nine specimens taken from untreated sea water at 21.5° and tested in a 14°–32° gradient spent, on the average, 74 per cent of the time in four adjacent compartments (22° to 28°). The mean selected temperature was 25.2°.

In experiments with *Fundulus parvipinnis* only specimens which had been kept for one to two months in the three constant-temperature tanks were used. This species inhabits shallow bays and sloughs, in which very high temperatures occur, and is more resistant to high temperatures than *Girella.* My specimens were originally taken from water at 27° in June, but temperatures several degrees higher are probably not uncommon in their habitat. While these fishes reacted to the gradient, their reactions were not nearly as sharp and consistent as those of *Girella.* They were frequently very inactive and "nervous," and showed such a strong tendency to move to the ends of the tank that it was not always possible to repel them from both ends, even though a steeper gradient was used. Nevertheless, a few interesting observations were made. In a 14° to 35.5° gradient, twelve 20°-conditioned and six 30°-conditioned specimens showed marked avoidance of the lower temperatures, but were not consistently repelled by the highest temperatures. In both cases about 75 per cent of the time was spent in the warm half of the gradient (26° to 35.5°). However, when the 20°-conditioned specimens were tested in an 18°–40°gradient, all avoided the warmest water. Seven 10°-conditioned specimens tested in a 14°–35° gradient were very inactive, but all avoided both ends of the gradient and spent 75 per cent of the time in four intermediate compartments (18.5°–26°).

Several bottom-living forms (*Gillichthys mirabilis* Cooper, *Leptocottus armatus* Girard, *Clinocottus analis* (Girard), etc.) were tested in temperature and salinity gradients. They were very inactive, and no marked reactions were observed. Two specimens of *Clinocottus,* a fish which is little resistant to dilute water, moved into such water and remained there until they died. A single young specimen of the blenny *Heterostichus rostratus* Girard was tested in a salinity gradient. This fish was quite active and showed a marked avoidance of diluted water.

Discussion and Conclusions

The ability of the more active fishes to avoid harmful temperatures and to exercise selection in an experimental temperature gradient has been demonstrated.

Fishes which had been taken from their normal environment could not be regarded as selecting temperatures to which they had been acclimatized. Differences of previous temperature history did not necessarily result in differences of behavior in the gradient. At most, the influence was only temporary. Thus, *Girella* which had been conditioned to 10° always moved into higher temperatures. Had they been left in the gradient, they would have become acclimatized to these higher temperatures and, in time, would have selected as high a temperature as specimens which had been kept continuously at 20° or 30°. The data in Fig. 5 indicate that a number of days would have been required for this change to take place. Obviously, selection in a gradient is not a reliable index or measure of acclimatization.

The term " optimum," as used to designate the most favorable condition for an entire organism (rather than for a single process or activity), has never been adequately defined. At any rate, a temperature " optimum " or " habitat preference " which changes with the temperature of the environment (and consequent acclimatization) is of questionable significance. However, the fishes did not select temperatures to which they were accustomed, but showed a marked tendency to move, in time, into a common range of temperatures. This range of temperatures must have some physiological significance. For the view that it represents an " optimum " or a " habitat preference," however, I find no evidence. The temperatures most frequently selected by normal *Girella* (26°–27°), even after they had been living in relatively cold water, are not normal in their habitat. The average surface temperature to which these fishes are exposed in this locality is 16.9° (Mean for 18 years). The mean temperature for August, the warmest month, is 20.8°, while the highest monthly mean on record (July, 1931) was 23.6°. Under these (suboptimal?) conditions the species has been living and reproducing successfully for an indefinite period.

The negative response to extreme and obviously harmful temperatures is unquestionably adaptive. The assumption that the specific behavior of animals in response to all other thermal stimuli must necessarily have similar significance is open to question. Granting, however, that the sharp avoidance reactions were given to "unfavorable" stimuli, the conclusion does not follow that the selected temperatures were more "favorable" or "preferable" except under the special (and somewhat unnatural) conditions of the experiment. It is important to distinguish between the excitatory or detrimental effects of given rapid *changes* of temperature and the effects of given temperatures upon an organism which is exposed to them continuously. Particularly is this so in the case of poikilotherms.

In my discussion of the behavior of *Girella* (based upon several hundred prolonged individual observations), I have shown that the fishes did not move directly into a certain temperature and remain there. Instead, the phenomenon of selection and the fluctuations of the selected temperature were shown to be the result of a succession of characteristic avoidance reactions (turning and swimming back) of varying intensity to *contrasting* temperatures encountered in the course of random movements. These reactions were subject to modification by rapid "adaptation." The correspondence between this behavior and the familiar effects of "adaptation" and "contrast" in human cutaneous thermal sensations is obvious.[8] From the observed reactions, it appears that a rapid change, for example, from 15° to 25°, is less stimulating and repellent than (and, perhaps, is preferred to) the reverse change following exposure to the higher temperature. It does not follow, however, that the lower temperature is not entirely as "favorable" (physiologically) or "satisfactory" (psychologically) as the higher one in the absence of contrasting temperatures. The fact that all species tested selected relatively high temperatures indicates that, in general, within normal ranges of temperature rapid cooling is more detrimental or stimulating than a rapid rise of temperature. Whether this difference depends upon peculiarities of the cutaneous thermal sense or has more general physiological significance is not clear. The temperatures selected by different species may be expected to bear some approximate relation to the maximal temperatures which they can tolerate and, consequently, to the temperatures which occur in their natural environments. Thus, *Fundulus* was not repelled by high temperatures which were strongly repellent to *Girella*. This is hardly evidence that an " optimum " was selected in either case.

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[8] This rapid "adaptation" should not be confused with the more gradually produced and lasting changes affecting behavior, resistance, metabolic rate, etc., which I have referred to above as acclimatization (See Sumner and Doudoroff, 1938, and citations).

Analogies between experimental gradients and those which occur in nature should be regarded with caution. The former are usually much steeper. Fishes in them are so confined that their random movements bring them repeatedly into widely different conditions at a rate with which " adaptation " can not keep pace. No correspondence between the reactions to these two types of gradient can be assumed.

The above conclusions probably apply, to a large extent, to reactions to other stimuli than temperature. Certainly, the relative vigor of the reactions of fishes to different stimuli can not be relied upon to show what characteristics are more important as indices of the suitability of environments. My results with temperature and salinity gradients, in which *Girella* selected entirely unnatural conditions, support this conclusion. They indicate only that the fishes were more sensitive to changes of temperature than of salinity. This difference may depend, largely, upon the nature of the receptive apparatus involved.

SUMMARY

An apparatus is described whereby a steep horizontal temperature (or salinity) gradient can be maintained, without the presence of vertical gradients, for the study of the reactions of fishes. The reactions of several species of marine fishes, chiefly of *Girella nigricans* (Ayres), to these gradients have been studied. A marked selection was demonstrated of temperatures which were relatively high in comparison with the normal environment of the fishes. The influence of acclimatization and of other factors upon temperature selection was investigated. While acclimatization was found to influence selection, the effect was slight and only temporary, the selected temperature being, to a large extent, independent of past experience. The physiological and ecological significance of selection in the experimental gradient is discussed. The rôle of contrast and of rapid " adaptation " is indicated. It is concluded that selection is indicative of the relative stimulative or detrimental effects of given rapid *changes* of temperature, while the common view that such selection indicates the nature of " optimal " conditions or habitat preference is not tenable.

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STUDIES IN THE PIGMENTARY SYSTEM OF CRUSTACEA. IV. THE UNITARY VERSUS THE MULTIPLE HORMONE HYPOTHESIS OF CONTROL

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Since the first demonstration by Perkins (1928) that the physiological activity of crustacean chromatophores is regulated by a hormone originating from the eye-stalks, numerous studies have confirmed and extended his results (for a review of the literature see Hanström, 1937). It was later shown (Kleinholz, 1934, 1936) that the retinal pigments of crustaceans were also under endocrine control, being affected by the same eye-stalk extracts which contracted[2] the chromatophores of the integument.

On the basis of independent chromatophoral response to various colored backgrounds, Brown (1935*a*) concluded that a number of different hormones were concerned in the regulation of color change. Abramowitz (1937*b*), on the other hand, has questioned the necessity of more than one hormone in explaining the pigmentary activity of crustaceans. When the evidence available from studies of the retinal and integumentary pigments is considered, it appears that at least two different hormones might be involved. For example, while the chromatophores of *Palaemonetes vulgaris* are maximally concentrated[2] and the distal retinal pigment is in the position of complete light-adaptation in animals kept on an illuminated white background, on an illuminated black background the red chromatophores are maximally dispersed but the distal retinal pigment is still in the light-adapted position. If both types of pigment cells were controlled by the same hormone, then absence of the chromatophorotropic hormone from the circulation (evidenced by the dispersed integumentary chromatophores in animals maintained on an illuminated black background) should also result in a position of the distal retinal pigment characteristic for complete dark-adaptation.[3] It is obvious, on the other hand, that the different behavior of these two components of the pigmentary system could be explained on the basis of differential threshold sensitivities to the same hormone.

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[1] Sheldon Travelling Fellow of Harvard University.

[2] The terms "contracted" and "expanded" in reference to the condition of the pigment within chromatophores are used synonymously with the concentrated and dispersed state.

[3] Kleinholz and Knowles (1938) have shown that the position of the distal retinal pigment is directly proportional to the ratio, $\frac{\text{intensity of incident light}}{\text{intensity of reflected light}}$.

Diurnal rhythms in the pigmentary system of crustaceans, persisting under constant external conditions, afford an opportunity to test the question of the unitary or multiple hormone hypothesis. Three crustaceans are known in which the integumentary chromatophores behave in such rhythmic fashion: a macruran, *Hippolyte varians* (Gamble and Keeble, 1900); an isopod, *Ligia baudiniana* (Kleinholz, 1937*a*); and a brachyuran, *Uca pugilator,* (Abramowitz, 1937*a*). When individuals of these three species are kept in constant darkness the animals become pale at night and darken again during the day. Welsh (1930*a*, 1935) and Kleinholz (1937*b*) have shown that similarly persistent cyclic activity of the retinal pigments occurs in macrurans and brachyurans when maintained under constant conditions of illumination. On the basis of the unitary hormone hypothesis the threshold of the retinal pigment must be lower than that of the integumentary pigments; persistent rhythm in the integumentary chromatophores of a crustacean should therefore also be accompanied by a persistent cyclical behavior in the activity of the retinal pigments. This paper reports the results from experiments designed to determine whether pigmentary activity in crustaceans is to be explained on the basis of one or of more than one hormone.

These studies were conducted in part at the Marine Biological Laboratory of the United Kingdom, Plymouth, England, and in part at the Stazione Zoologica, Naples, Italy. To the Directors of these laboratories, Dr. Stanley Kemp and Professor R. Dohrn, and to the staffs of these institutions I am greatly indebted for generous facilities and kind assistance. I am also obliged to the committee for permission to occupy the Jacques Loeb Memorial Table at Naples.

The crustaceans used in these studies were *Crangon vulgaris, Hippolyte varians,* and *Leander adspersus.*

Materials and Methods

Black- and white-painted dishes were used as backgrounds to obtain individuals with expanded and contracted melanophores (*Crangon*); these vessels were illuminated from above by a 60-watt electric lamp at a distance of 30 inches.

It was desirable in some experiments to observe the color reactions of blinded individuals.[4] Two methods were used to incapacitate the retinas of *Crangon:* in one, an opaque mixture of plaster of Paris was moistened into a paste and spread over the entire eye-stalk, while in the second the retinal portion of the eye-stalk was destroyed by cautery. In removing the eye-stalks of *Crangon* it was found advisable to proceed in two stages, allowing a day or two to elapse before ablation of the second eye-stalk.

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[4] Blinding may be of two kinds (Kleinholz, 1937*a*); incapacitating the retina, whereby only the sensory elements are affected, and total ablation of the eye-stalks, resulting in removal of the chief functional source of eye-stalk hormones.

Blood "transfusions" from black-background-adapted [5] individuals to white-background-adapted [5] shrimps and *vice-versa* were effected by withdrawing blood from the pericardial sinus of donors (approximately 0.1 cc. could thus be obtained from a single individual) by means of a hypodermic syringe, and injecting 0.05–0.07 cc. into the abdominal musculature of the recipients.

Extracts of the rostral region of *Crangon* were prepared in an attempt to duplicate the results of Koller (1928). Both eye-stalks of the donor were first ablated at the base to avoid the possibility of including fragments of Hanström's sinus gland; the rostral region was then excised with a second pair of scissors as indicated in Fig. 1 of Koller's (1928) paper. The excised portions from 10 black-adapted *Crangon* were triturated for 3 minutes in 1.0 cc. of filtered sea water or crustacean perfusion fluid (Pantin, 1934). One-tenth cc. of the clear supernatant extract was injected into the abdominal musculature of the white-adapted recipients which were then returned to their white backgrounds for observation. Precautions were taken to prevent contamination of the fluids used for injection; separate syringes and needles were used in taking blood from white- and from black-adapted individuals; a third hypodermic syringe was used exclusively for injecting extracts prepared from the rostral regions. Before use the syringes were washed in tap-water, immersed in bichromate-sulfuric acid solution for 12 hours, flushed in running tap-water for 12 hours, rinsed several times in distilled water, 95 per cent alcohol, ether, and dried in air.

Destruction of the rostral region, claimed by Koller to be the source of a melanophore-dispersing hormone, was performed by cautery in the manner prescribed by him (1928). The exoskeleton in the region indicated on his Fig. 1 was excised and a hot needle used for surface and deep cautery; the replaced square of excised exoskeleton was usually kept in position by the blood clot which formed around the edges; in some cases coagulation of the blood exuding around the wound was hastened by drawing the cautery needle along the margins of the incision.

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[5] The terms "black-background-adapted," "black-adapted" and "black" are used to describe individuals in which the melanophore pigment has been dispersed as a result of the animal's having been kept on an illuminated black background; "white-background-adapted," "white-adapted," and "white" are similarly used to describe the concentrated condition of the melanophores in animals kept on an illuminated white background.

Hippolyte varians which had been kept in darkness and in light were killed in hot water (80° C.) during the day (" day light " and " day dark " eyes) and at night (" night light " and " night dark ") to determine whether a persistent diurnal rhythm existed in the movements of the retinal pigments. Subsequent histological treatment of the eyes followed the method described in an earlier paper (Kleinholz, 1937*b*). Blinding operations, similar to those performed on *Crangon,* were made on *Hippolyte;* retinas were destroyed by cautery or were ablated with iridectomy scissors; entire eye-stalks were removed after first crushing the base to prevent excessive hemorrhage.

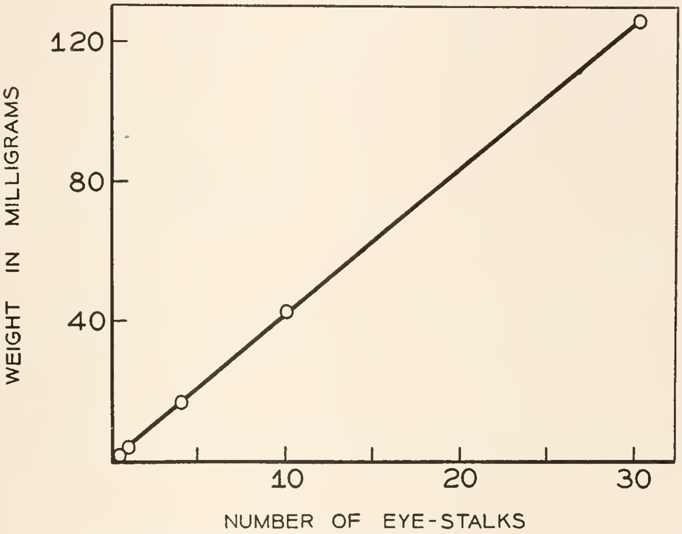

FIG. 1. The relation between the number of eye-stalks and their " wet weight " in milligrams.

Leander adspersus was used in the quantitative studies for the determination of threshold concentrations of hormones for the retinal and integumentary pigments. The procedure was standardized; stalk extracts were prepared from animals which measured exactly 1.9 cm. in the midline from the tip of the rostrum to the posterior margin of the cephalothorax. This proved the most accurate method of assuring the use of eye-stalks of the same size. Earlier attempts to measure the eye-stalk itself were unsatisfactory. That the method used was justified is shown by the accompanying curve (Fig. 1) in which the relation between the number of eye-stalks and their " wet " weight (the stalks were rolled over filter paper to remove excess moisture) is plotted. It is reasonable to expect that Hanström's sinus gland or whatever glands are responsible for the source of the pigment-activating hormones would show a similar relation to the size and number of eye-stalks used in preparing the extract.

The stalks were excised from animals which had been kept on an illuminated white background, thereby insuring maximum hormone content (Kleinholz, 1936; Abramowitz, 1937*a*), and were triturated in a small mortar with 0.5 cc. of cold-blooded Ringer's solution. The resulting tissue suspension was brought to a boil and Ringer's solution added to make 1.0 cc. of clear extract which could be diluted to desired amounts. Fresh preparations were made for each daily series of experiments. Exactly 0.04 cc. of extract were injected into the abdominal musculature of test animals.

Two different experimental conditions held in the determination of minimal thresholds for the retinal and body pigments. In all cases, however, the test animals were of the same size, 1.6 cm. from the tip of the rostrum to the posterior margin of the cephalothorax, as measured along the mid-dorsal line. Despite this precaution to assure the use of a standard test object, there were slight variations in the responses of the retinal pigments which must be attributed to individual differences between animals.

The responses of the distal retinal pigment to injection of eye-stalk extract were measured in animals which had been adapted to darkness overnight. The method used was a modification of that first described by Welsh (1930*b*). Four prawns were placed in individual 500-cc. beakers, containing about 200 cc. of sea water, which were then transferred to a dark-box in a photographic dark-room. An ordinary compound microscope, fitted with a micrometer ocular, was used to measure the position of the distal retinal pigment in the dark-adapted animals. The source of illumination was a sub-stage microscope lamp over the face of which had been fitted a light-mask with a circular opening 1 cm. in diameter. The intensity of the light was further reduced by using a blue glass filter. Preliminary trials showed that no proximal migration of the distal pigment occurred in the eyes of dark-adapted test animals when they were kept for as long as 20 minutes in the rays of this lamp.

The cell was made of colorless celluloid, 4 inches square, with two openings in the walls to allow circulation of sea water. The bottom of the cell was covered to a depth of 1 cm. with a black paraffin mass, except for a free circular area for light to pass into the microscope and a shallow trough to receive the body of the experimental animal. A prawn could be kept in position, with its head projecting over the opening, long enough for a reading to be made by placing a small glass slide on the telson and another on the rostrum. After each reading the animal was returned to its beaker.

In determining the minimal threshold of the body pigment to eye-stalk extracts, standard animals from which both stalks had been previously removed were used. Although *Leander* possesses red, yellow and white chromatophores, only the red ones were watched closely in their responses to stalk extract. Observations of the chromatophores were made by means of a binocular dissecting microscope.

Background Responses of Crangon

Koller's studies of chromatophoral activity in *Crangon vulgaris* were the first to present evidence in favor of the multiple hormone hypothesis in the pigmentary behavior of crustaceans. He reported (1927) that blood from dark individuals, when injected into white-adapted *Crangon,* resulted in darkening of the recipients. After cauterizing the rostral region and injecting extracts prepared from tissue of this region (1928) he concluded that an organ in this vicinity was the source of the melanophore-dispersing hormone which he called "expantin." He also confirmed Perkins' results and named the chromatophore-concentrating principle "contractin." Various American investigators (Perkins and Snook, 1931; Kropp and Perkins, 1933; Brown, 1935*a*) were unable to confirm the presence of a chromatophore-dispersing hormone in the crustaceans they studied. Beauvallet and Veil (1934) reported a slight darkening in some cases when *Palaemon squilla* were injected with large doses of rostral extract. This section of the present study is a description of results obtained in an investigation of the reported melanophore-dispersing hormone in *Crangon vulgaris.*

The accompanying Table I is a summary of the responses obtained in these experiments. It is evident that, in normal animals, the melanophore pigment is concentrated on an illuminated white background and maximally dispersed on a black one and in darkness. In individuals whose retinas had been opaqued or destroyed by cautery, the melanophores were always maximally dispersed, regardless of the background on which the animals were kept, resembling in this respect those of shrimps maintained in total darkness. These results eliminate the possibility of a direct response of the melanophores to light and to darkness. The behavior of the melanophores in *Crangon* from which the eye-stalks had been removed is slightly different; instead of the maximum dispersion, such as found in normal shrimps kept in darkness and in individuals with incapacitated retinas, the melanophores of stalk-less animals

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were in a condition intermediate between concentration and dispersion. Such individuals, although darker than *Crangon* adapted to white backgrounds, were readily distinguishable from those with incapacitated retinas (Fig. 5).

The white chromatophores behaved quite differently. The guanophores were dispersed in all *Crangon* (normals, individuals with both eye-stalks ablated, and those with incapacitated retinas) which were maintained in an illuminated environment, regardless of the color of the background, and were concentrated in those shrimps kept in total darkness. Similar results were reported by Perkins (1928) and by Hanström (1937) for *Palaemonetes vulgaris* and by Stephenson (1934) for *Leander serratus*. Stephenson and Hanström explained this as a direct response of the guanophores to light and to darkness, although the latter investigator admitted the possibility of a reflex connection between additional photoreceptors (a persistent nauplius eye, Hanström, 1937) and secondary sources of a guanophore-contracting hormone (Brown, 1935*a*). Experiments designed to test these possibilities have been performed by Mr. F. G. W. Knowles of Oxford University and will be published elsewhere.

TABLE I

The responses of the chromatophores of *Crangon vulgaris* to various conditions of illumination. *E.S.*, both eye-stalks ablated; *R.C.*, both retinas cauterized; *R.O.*, both retinas opaqued; *C*, pigment concentrated; *D*, pigment dispersed; *I*, pigment intermediate between concentration and dispersion; *Irreg.*, pigment irregular; (*K*), results obtained by Koller.

Illumination	Condition of animals	Chromatophore pigment	
		Black	White
White background	Normal	*C*	*D*
White background (*K*)	Normal	*C*	*D*
White background	*E.S.*	*I*	*D*
White background	*R.C.*	*D*	*I–D*
White background	*R.O.*	*D*	*D*
Black background	Normal	*D*	Irreg.
Black background (*K*)	Normal	*D*	*C*
Black background	*E.S.*	*I*	*D*
Black background	*R.C.*	*D*	*D*
Darkness	Normal	*D*	*C*
Darkness	*E.S.*	*I*	Irreg.
Darkness	*R.C.*	*I*	*C*
Darkness	*R.O.*	*D*	*C*

THE RÔLE OF THE SO-CALLED "ROSTRAL ORGAN" IN THE CHROMATIC PHYSIOLOGY OF THE MELANOPHORES IN CRANGON

Koller (1927) reported that blood of a black *Crangon,* when "transfused" into one in which the melanophores were contracted, induced dispersion of the melanophore pigment within 30 minutes. This led him to postulate the presence of a melanophore-dispersing hormone, the source of which he later (1928) believed to be an organ in the rostral region.

The results of my experiments to test for the presence of a melanophore-dispersing principle are summarized in Table II. When 43 white-adapted individuals were injected intramuscularly, each with 0.05 cc. of blood from black *Crangon,* only 27 per cent of the recipients darkened to an appreciable extent. In a control series, blood from white-adapted *Crangon* was injected in similar fashion into 54 white-adapted individuals; a like proportion, 22 per cent, darkened. The results indicate that the darkening induced by blood transfer is a non-specific response. This belief gained further support from the following observations: injection of extracts of the rostral regions, prepared from dark individuals, into 38 white-adapted *Crangon* induced darkening in only slightly over half the number of animals; filtered sea water and crustacean perfusion fluid were able in some cases to effect dispersion of melanophore pigment when injected into white-adapted individuals.

TABLE II

Results from injecting blood and control solutions into white-adapted *Crangon. Bb,* blood from black-adapted individuals; *Bw,* blood from white-adapted individuals; *Rb,* extract of rostral region from black-adapted shrimp; *S,* filtered sea-water; *P,* crustacean perfusion fluid (Pantin, 1934). A.M., experiments run in the morning from 9–12 o'clock; P.M., experiments run from 2–10 o'clock in the afternoon and evening.

Substance injected	A.M.			P.M.			Summary		
	Number injected	Number darkening	Number not changing	Number injected	Number darkening	Number not changing	Total injected	Total darkening	Total not changing
Bb.....	19	1	18	24	11	13	43	12	31
Bw.....	24	2	22	30	11	19	54	13	41
Rb.....	15	4	11	23	18	5	38	22	16
S......	—	—	—	10	8	2	10	8	2
P......	10	0	10	10	2	8	20	2	18

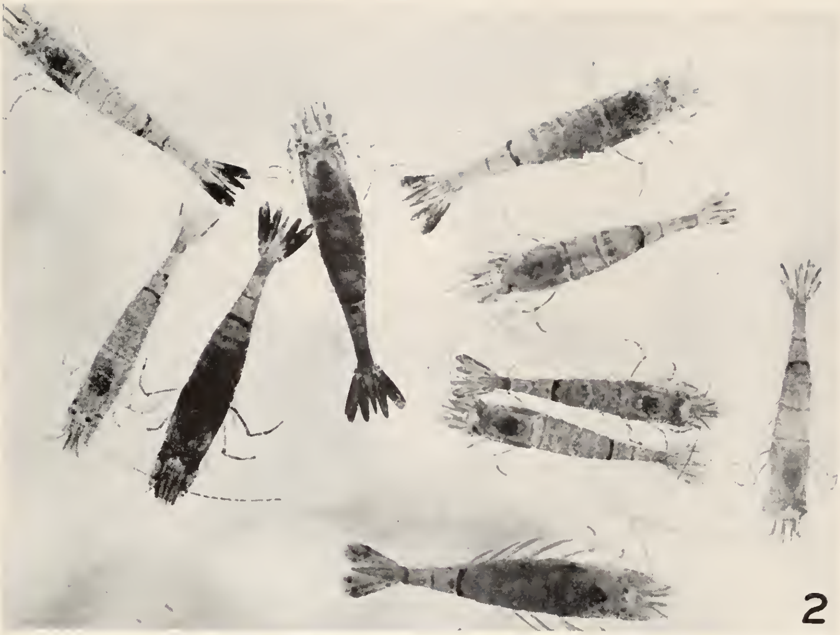
An apparent difference in the facility with which dispersion of the melanophore pigment could be induced was noticed between injections which were made in the morning and those which were performed in the afternoon and evening. No explanation for this situation is offered at present. During the period these experiments were performed (July to September), the pigmentary behavior of animals on the black and white backgrounds was perfectly normal all through the day; if the results from morning and evening injections are due to a cyclical release of some metabolite (or hormone) into the circulation as evening approaches, the normal chromatic behavior of individuals on white backgrounds gave no external indication of such internal changes.

The results of blood transfer in these experiments are thus seen to differ greatly from those reported by Koller. The latter author found that blood from dark individuals when injected into 46 white-adapted *Crangon* induced melanophore dispersion in 95 per cent of the cases, while blood from white-adapted individuals, used as a control, had almost no effect (1927, pp. 237–239).

To locate the origin of the melanophore-dispersing principle, Koller (1928) attempted local destruction of the regions suspected of this endocrine function. He reported that superficial cautery of a delimited area in the rostral region resulted in a permanent loss of the animal's ability to adapt to black backgrounds; *Crangon* operated in this manner remained light in appearance because of a complete concentration of the melanophore pigment.

My attempts to duplicate Koller's experiments were unconvincing in their results. In none of the 16 *Crangon* in which cautery of the rostral region was superficial did the melanophores become permanently and maximally concentrated; within a few minutes after the operation the dispersed melanophore pigment of black-adapted individuals became temporarily concentrated to an intermediate condition, but the pigment granules soon became dispersed again. Different results were obtained by deep cautery of the same region: of 69 shrimps thus treated, 9 became permanently pale, the melanophores being maximally contracted regardless of the fact that the animals were maintained on an illuminated black background (Fig. 4). *In each of these 9 individuals, however, equilibratory and swimming movements were grossly abnormal, the animals performing circus movements when swimming, and resting ventral side uppermost on the bottom of the container.*

The results from these experiments, while partly confirming those of Koller, do not lend ready support to his belief in the presence of two hormones. In the tests described above, the results from blood "transfusions" are seen to be too irregular to accept as evidence in favor of a melanophore-dispersing hormone in the circulation. The total ineffectiveness of superficial cautery of the rostral region, and the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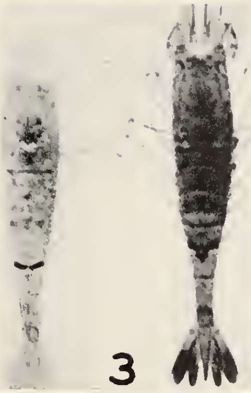
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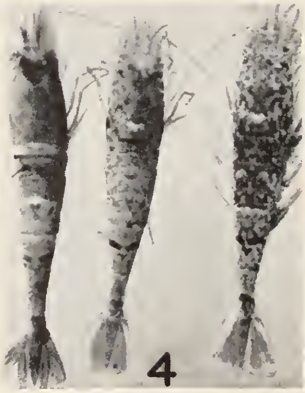
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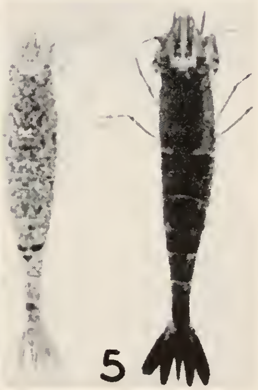
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PLATE I

The color reactions of *Crangon vulgaris.*

Fig. 2. Extract was prepared by triturating the rostral regions from 10 black *Crangon* in 1.0 cc. of filtered sea water; 0.05–0.07 cc. were injected into each of 10 white-adapted shrimps at 9:00 P.M. One hour later the maximum effect was recorded by this photograph.

Fig. 3. The normal color responses to white (on the left) and black (on the right) backgrounds.

Fig. 4. The two animals on the left were individuals in which the rostral region had been deeply cauterized, resulting in permanent pallor; both eye-stalks had been removed from the shrimp on the right, resulting in an intermediate condition of the melanophores.

Fig. 5. Both eye-stalks were removed from the animal on the left. Both retinas were destroyed by cautery in the specimen on the right; as a consequence the melanophores were completely dispersed.

fact that deep cautery in the same region resulted in only 13 per cent positive results must also be regarded with caution. The few cases of " successfully " cauterized animals were abnormal in their equilibrium and swimming movements, indicating injury of the central nervous system possibly accompanied by interference in the regulation of Hanström's sinus gland.[6] More critical evidence will be needed before the existence of a melanophore-dispersing hormone, originating in the rostral region, can be unqualifiedly accepted.

Persistent Cyclic Activity in the Pigmentary System of Hippolyte [7]

As mentioned briefly in the introductory section, the existence of persistent cyclic activity in the pigmentary system of various crustaceans when the individuals are maintained under constant environmental conditions, presents an opportunity to subject to critical test whether one hormone or more than one hormone is involved in the regulation of these effectors. Gamble and Keeble (1900) first reported a periodic contraction of the chromatophores in *Hippolyte varians* when these animals were maintained under constant conditions of illumination. It was decided to test the validity of the unitary hormone hypothesis, as presented by Abramowitz (1937*b*), by examining the positions of the retinal pigments in this animal under the various experimental conditions previously used in this procedure (Welsh, 1935; Kleinholz, 1937*b*). According to the unitary hormone hypothesis, the threshold for the

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PLATE II

Ommatidia from the eyes of *Hippolyte* to show the positions of the retinal pigments. *DL*, " day light " eye; *DD*, ommatidium from " day dark " retina; *NL*, " night light " retina; *ND*, unit from a " night dark " eye; *D*, distal retinal pigment; *R*, reflecting pigment; *P*, proximal pigment; *B*, basement membrane.

Figs. 6 to 9. Ommatidia from the retinas of *Hippolyte varians*. The pigments are in the positions characteristic for adaptation to light and to darkness; there is no persistent diurnal rhythm. Note that the reflecting pigment migrates in two directions in the light-adapted eye; in one, it forms a small cap above the distal retinal cells and in the other it moves below the basement membrane.

Fig. 10. " Day light " eye of *Hippolyte pleuracantha*. The three retinal pigments are in the typical light-adapted positions. The reflecting pigment is different from that in most prawns hitherto studied in that it has migrated almost wholly above the distal retinal cells instead of below the basement membrane.

Fig. 11. " Day dark " eye showing the persistence of diurnal rhythm. The reflecting and proximal pigments are in the dark-adapted position but the distal retinal cells remain in the position normal for a light-adapted retina.

Figs. 12 and 13. " Night light " and " night dark " ommatidia. These show the ordinary responses to light and to darkness.

[6] Bethe (1898) and Roeder (1937) have shown that the supraesophageal ganglion in arthropods exercises an inhibitory influence on many reflex activities.

[7] A preliminary report describing the results of part of the material included under this section has been published in collaboration with Dr. J. H. Welsh (1937).

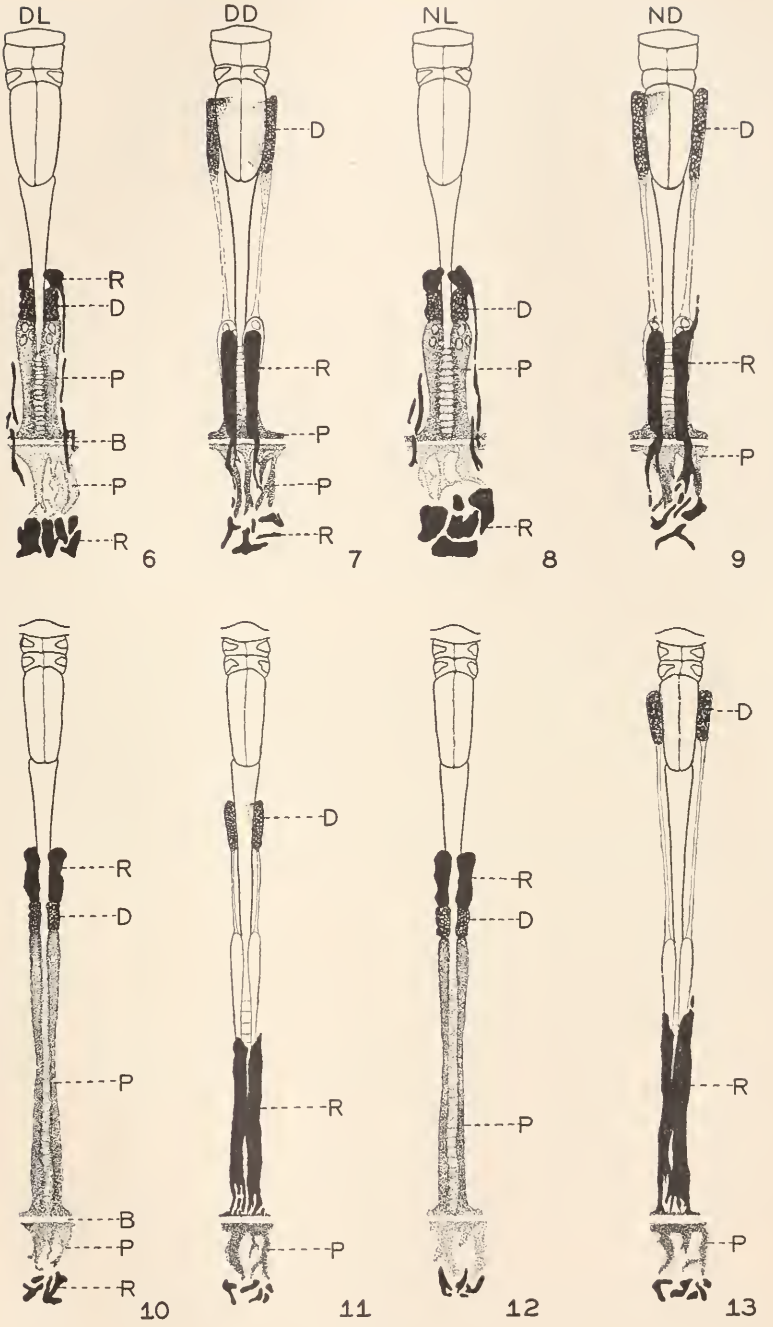
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PLATE II

distal retinal pigment must be lower than that for the integumentary chromatophores (Abramowitz, 1937*a,* p. 356). Consequently, a persistent cyclic activity of the chromatophores in *Hippolyte* should also be accompanied by similar activity of the retinal pigments.

When eyes of *Hippolyte varians* were sectioned and examined, no evidence could be found of a persistent rhythm; the retinal pigments (Figs. 6–9) were in the positions normally characteristic for adaptation to light and to darkness. This contradicts the unitary hypothesis. Through the generosity of Dr. J. H. Welsh, I was allowed to study and describe his preparations of retinas of another member of the same genus, *Hippolyte pleurocantha,* collected in Bermuda (on *Sargassum*). These showed a persistent rhythm, but not of the kind expected in *Hippolyte varians;* Gamble and Keeble (1900) stated that *H. varians,* when kept in continued darkness, became pale at night and remained dark during the day; under the unitary hypothesis the distal retinal pigment should be in the light-adapted position in " night dark " eyes; instead, the distal retinal pigment was in the typical light-adapted position in individuals, maintained in constant darkness, which had been killed during the day (Figs. 10–13).

Gamble and Keeble's study of color changes in *Hippolyte* led them to conclusions essentially similar to those held by many of the earlier investigators of metachrosis, namely, that any particular condition of the chromatophores was due to nervous impulses passing to them from the central nervous system. Because of the differing responses of the retinal pigments between the two species of *Hippolyte* described above and the unexpected difference between the periodical behavior of the integumentary and retinal pigments, a reinvestigation of the color changes of *Hippolyte varians* with regard to endocrinal control and persistent periodicity was undertaken.[8]

According to Gamble and Keeble (1900, p. 614) adult *Hippolyte* are capable of only very slow color changes on colored backgrounds. On close examination most individuals were refractory, and those which did change were slow and often irregular in their adaptations to black and to white backgrounds.[9] This is illustrated in the following protocol which is typical for several experiments:

Sept. 1.	10.30 P.M.	Four animals were placed on an illuminated black background.
Sept. 2.	10.45 A.M.	All are dark brown; the pigment in the red chromatophores is maximally dispersed. Animals transferred to an illuminated white background.

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[8] Only the chromatophores containing red pigment were studied.

[9] Minkiewicz (1908), however, reported that *Hippolyte varians* underwent adaptive color changes in response to changes in background.

12.10 P.M. One animal is green (chromatophores are punctate); the remaining three are light brown (chromatophores are dispersed).
4.00 P.M. All four prawns are brown with the chromatophores dispersed.
10.15 P.M. All four are yellowish-olive in color but the red chromatophores are maximally dispersed. The lighter tint must be due to a change in the yellow chromatophores which can't be seen clearly.

Injection of crustacean eye-stalk extracts into normal dark *Hippolyte* effects a rapid concentration of the pigment within the red chromatophores, accompanied by a diffusion of blue pigment into the surrounding tissues.[10] The same chromatophoral response can be elicited by injection of extracts prepared from the stalks of a different species; doses of 0.01–0.03 cc. of a preparation containing 5 eye-stalks from *Hippolyte* triturated in 0.2 cc. of sea water as well as similar doses of a preparation of 2 eye-stalks of *Leander serratus* in 0.5 cc. sea water, when injected into test *Hippolyte,* brought about the same chromatic condition.

Normal *Hippolyte,* as well as those from which the retinas had been removed, were examined to see whether the cyclic activity reported by the English authors persisted. The following summaries show that this nocturnal condition is independent of the retinal receptors:

A. Normal individuals with intact retinas
Sept. 9. 5.30 P.M. Nine animals placed in darkness.
10.45 P.M. All are complete or partial nocturnes. In the latter the chromatophores are punctate or slightly stellate in the anterior half up to the " hump " or point of flexure of the tail; those of the telson are dispersed.

B. Animals with both retinas removed
Aug. 27. 11.30 P.M. Seven prawns placed in darkness.
Aug. 30. 5.00 P.M. All are nocturnes, the red chromatophores being punctate.
Sept. 1. 12.30 A.M. In five prawns the chromatophores are punctate, in two they are stellate.
10.00 P.M. All 6 survivors are nocturnes.

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[10] Gamble and Keeble (1900) have called this chromatic condition " nocturne " because they found it appearing in normal animals each evening as darkness fell.

In all reports dealing with metachrosis in the macruran crustaceans, removal of both eye-stalks has resulted in a permanent darkening of the animals due to the dispersion of the chromatophoral pigments; this results because of loss of the sinus glands, the source of the chromatophore-concentrating principle. It therefore follows that, if the nocturnal color state is effected by release of the eye-stalk hormone, removal of both eye-stalks should prevent the appearance of this chromatic condition in animals thus operated. The following summary shows such an assumption to be incorrect:

C. Animals with both eye-stalks removed

Aug. 30.	10.00 A.M.	One eye-stalk removed from each of 10 *Hippolyte;* animals placed in darkness.
	11.15 P.M.	All 10 are nocturnes; the red chromatophores are punctate.
Aug. 31.	11.00 A.M.	The second eye-stalk removed from each; 7 survivors were returned to the dark box.
Sept. 1.	12.15 A.M.	3 are nocturnes; chromatophores are punctate. 2 are light brown; chromatophores are intermediate. 2 are brown; chromatophores dispersed.
	10.00 P.M.	In 3 the chromatophores are punctate; in 3 they are intermediate; and in 1 they are dispersed.
Sept. 2.	10.25 P.M.	4 are partial nocturnes as in "B" above; in 1 the chromatophores are intermediate; and in 2 they are irregular between the intermediate and dispersed state.

Brown (1935*a*) found that injection of extracts of the central nervous system (exclusive of the eye-stalks) concentrated the chromatophoral pigments and has cited the persistence of the chromatic rhythm in *Hippolyte varians* from which both eye-stalks have been removed as additional proof of the existence elsewhere than in the eye-stalks of glandular tissue capable of secreting the chromatophore-concentrating principle. At present this view is not conclusively supported by experimental evidence. For, in spite of Brown's results and the report by Hanström (1937) that a persistent nauplius eye is present in *Palaemonetes* (thus presenting the possibility of a functional photoreceptor with reflex connections to a source of the chromatophore-concentrating principle), there is no positive physiological proof that such a system is able to function. In fact, the available evidence contradicts this possibility; *Palaemonetes* from which both eye-stalks have been removed lose their ability to adapt to changes from black to white backgrounds and remain permanently dark because of the dispersion of their chromatophoral pigments.

It is not necessary to consider the results presented in the above summaries on *Hippolyte* as due to a functional reflex between photoreceptors and glands; the nocturnal coloration of *Hippolyte* can be more simply explained as due to a direct effect of darkness on the pigment cells. Thus, these prawns would be dark in color when illuminated and would be nocturnes when placed in darkness. Were it not for the persistent cyclic activity of the chromatophores described by Gamble and Keeble, the accounts of chromatophoral behavior presented above would confirm this point. Normal *Hyppolyte* and individuals whose retinas and eye-stalks had been removed were therefore placed in darkness and examined several times during the day to eliminate the effects due to the reported periodic phenomenon. The chromatic condition of the operated and normal animals was always the same in individuals which had been kept in darkness; the prawns were either total or partial nocturnes with the chromatophoral pigments concentrated. When such pale animals were removed from the dark-box and placed in diffuse sunlight or under the rays of a 60-watt electric lamp they became completely dark within 10 minutes; microscopic examination revealed maximum dispersion of the chromatophores. These results indicate that the integumentary chromatophores of *Hippolyte* can respond directly to changes in light-intensity. Gamble and Keeble's observations of persistent cyclic activity of these effectors are not confirmed. Their report that these prawns remained dark during the day when kept in darkness and paled only as night fell seems probably due to the fact that light conditions in their experiments were not constant.[11]

THRESHOLD CONCENTRATIONS OF EYE-STALK HORMONE FOR RETINAL AND INTEGUMENTARY PIGMENTS IN LEANDER ADSPERSUS

The method of analysis used with *Hippolyte* having proved inconclusive, it was decided to measure actual threshold concentrations of the eye-stalk extract for the retinal and integumentary pigments. The standardization of the procedure has been described under "Methods." The data for the responses of the distal retinal pigment to various concentrations of eye-stalk extracts are arranged in Table III and are plotted in the curves shown in Fig. 14. It can be seen that injection of 0.04 cc. of an extract containing 0.1 eye-stalk in 1.0 cc. of Ringer's solution is one which gives a slightly perceptible response of the distal retinal pigment. More dilute extracts and control injections of Ringer's solution effected no discernible migration of this pigment over a period of 30 minutes. This concentration of stalk extract can therefore be labelled the minimal effective dosage for the distal retinal pigment. This is equivalent to 0.016 mg. (wet weight) of eye-stalk.[12]

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[11] To obtain the condition of constant darkness they covered the vessels in which their animals were confined with a double thickness of black cloth; these containers may not have been completely light-proof.

TABLE III

The responses of the distal retinal pigment in animals injected with various concentrations of eye-stalk extract. The concentration is expressed in the fraction or number of eye-stalks in 1.0 cc. of cold-blooded Ringer's solution; the distance migrated in micra is the average for the total number of eyes studied.

Conc. of extract	0.1	0.2	0.5	1.0	4.0	10.0	15.0
No. measured	16	12	18	12	6	6	6
Minutes after inj.							
0	0 μ	0 μ	0 μ	0 μ	0 μ	0 μ	0 μ
15	19	15	40	79	87	73	100
30	22	46	73	104	150	137	—
35	—	—	—	—	—	—	156
45	17	41	73	100	159	—	171
50	—	—	—	—	—	171	—
60	14	36	49	96	139	—	—
65	—	—	—	—	—	—	170
75	14	22	28	90	132	—	—
80	—	—	—	—	—	174	—
90	—	21	28	61	105	—	—
95	—	—	—	—	—	—	152
105	10	18	27	36	66	—	—
120	—	—	24	24	57	—	—
125	—	—	—	—	—	109	—
135	—	—	14	—	—	—	112
140	—	—	—	—	13	—	—
155	—	—	—	—	—	61	—
165	—	—	5	—	—	—	73
195	—	—	—	—	—	—	59

[12] Abramowitz and Abramowitz (1938, p. 281), after making several assumptions, find the effective dosage for the melanophores of *Uca* to be 0.000016 gamma of hormone. By making the same assumptions the minimal threshold for the distal retinal pigment of *Leander* is 0.0008 gamma and that for the red chromatophores is 0.00004 gamma. It is probably preferable, however, in describing effective dosages, to express these in terms of dry weight of eye-stalk.

The curves show that with increasing concentration of stalk extract the amount of migration of the distal retinal pigment increases until a maximum is reached (between 4 and 10 eye-stalks per 1.0 cc.). This is the maximum threshold concentration; although more concentrated extracts do not affect the amount of migration of the distal retinal pigment, there is some indication that "recovery" or return of distal pigment cells to the position characteristic for the dark-adapted eye is further delayed; this situation is probably dependent on the rate of elimination or destruction of the injected hormone.

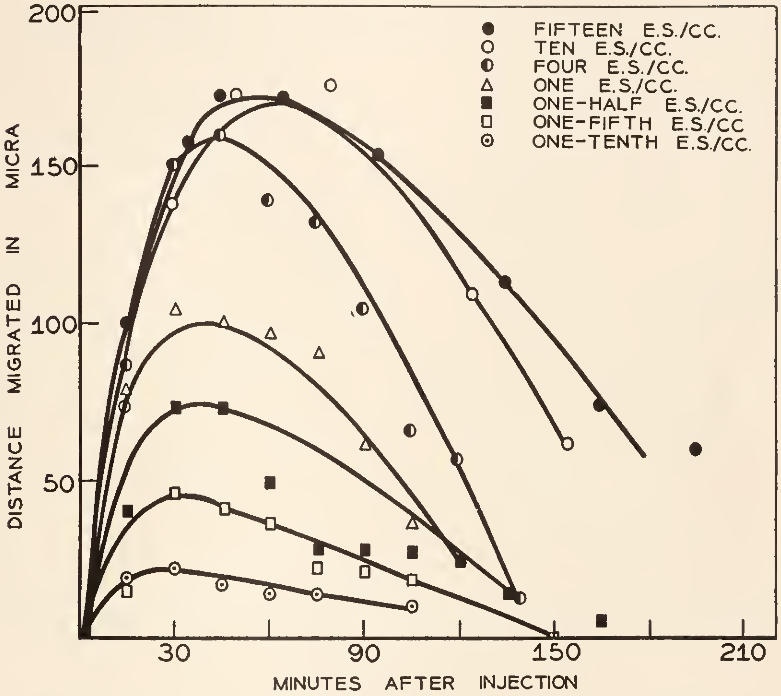

Fig. 14. The rate of migration of the distal retinal pigment cells in dark-adapted *Leander adspersus* in which have been injected various concentrations of eye-stalk extract. The distance migrated was measured from the cornea to the distal margin of the pigment cells.

The relation between amount of migration of the distal retinal pigment and the concentration of the injected eye-stalk extract is shown better in Fig. 15, where the distance migrated in micra (obtained from the curves in Fig. 14) is plotted as a function of the logarithm of the concentration, 30 minutes after injection. At this (and at a 45-minute) interval the relation is a direct proportion until the maximum migration is attained at the maximum threshold concentration, the effect of supramaximal concentrations being reflected in the levelling-off of the curves. This is in agreement with the known observations (Welsh, 1930*b*; Kleinholz, 1936) that migration of the distal retinal pigment to the light-adapted position requires between 30 and 45 minutes. A direct proportion is also shown when similar curves are drawn for intervals 15 and 75 minutes after injection.

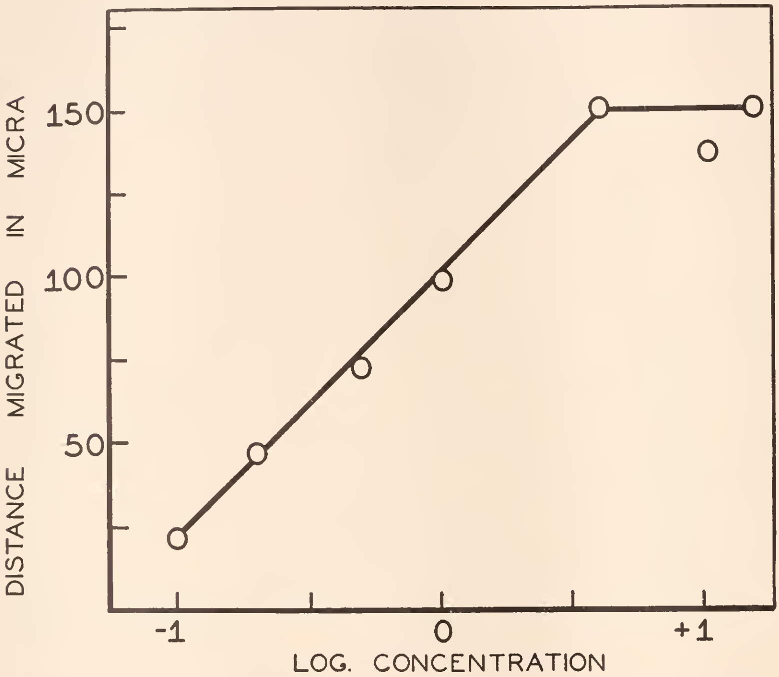

FIG. 15. The relation between migration of the retinal pigment and the concentration of the injected stalk-extract. The values for the position of the pigment were calculated from Fig. 14.

In determining the minimal threshold of the integumentary chromatophores, groups of 5 standard prawns which had been blinded a week previously by amputation of both eye-stalks were each injected with 0.04 cc. of the prepared stalk-extracts. With concentrations of 1.0, 0.5, and 0.2 eye-stalks per 1.0 cc. there was maximum concentration of the chromatophoral pigment within 5 minutes after injection, accompanied by the appearance of the characteristic diffuse blue coloration; the erythrophores of animals treated with these extracts remained punctate for 1 hour or more before the stellation indicative of approaching pigment dispersion was discernible. When concentrations of 0.05, 0.02, and 0.01 eye-stalks per 1.0 cc. were used, the chromatophores became punctate within 5 minutes but darkening began between 30 and 40 minutes after injection in each of the 5 test prawns in a series. In the final series 5 *Leander* (both eye-stalks amputated) were injected standard doses of extract containing 0.005 stalks per 1.0 cc. of Ringer's solution; ten minutes after injection the chromatophores in 2 of the prawns were completely punctate, in 1 animal they were variable between punctate and stellate, and in the remaining two the chromatophores ranged from partial stellation to complete dispersion. Thirty minutes after the injection darkening of the animals was evident; at the end of 1 hour four of the five animals were dark while the fifth was still intermediate in color. Control injections of cold-blooded Ringer's solution had no effect on the chromatophoral pigments. Injection of extracts of this concentration into normal prawns maintained in darkness had no discernible influence on the position of the distal retinal pigments. This concentration (0.005 eye-stalks per 1.0 cc.) is equivalent to 0.0008 mg. wet weight of eye-stalk and represents the minimal threshold for the integumentary chromatophores. The lower threshold of the chromatophores is thus about one-twentieth that of the distal retinal pigment.[12] These values do not support the unitary hormone hypothesis which requires that the threshold for the retinal pigment be lower than that for the body chromatophores.

Additional physiological evidence shows that the retinal and integumentary effectors react differently to darkness. In most macruran crustaceans thus far studied, the retinal pigments of animals maintained in darkness are found in the position characteristic for that state (except in those cases where a persistent cyclic activity affects one or more of these pigments); this is also true of *Leander adspersus*. In total darkness, however, the red chromatophores of this prawn are completely punctate and the animal is pale in appearance; similar chromatophoral behavior was noticed with *L. serratus*. This response is not a direct effect resulting from the exclusion of light, for individuals from which both eye-stalks had been removed and which had darkened in consequence remained unchanged in color when kept in total darkness. The concentrated state of the red chromatophoral pigments in darkness is therefore dependent upon release of the chromatophore-contracting principle from the eye-stalks. The importance of this situation is fully recognized by Abramowitz (1937*a*, p. 363): "The key to the whole situation lies in the behavior of the red and yellow chromatophores in animals maintained in darkness. If Brown is correct in noting that the more usual condition of the red and yellow pigments of animals maintained in darkness was slight dispersion, and that a long sojourn in darkness resulted in the same condition of these pigments as that occurring when animals were adapted to a red background, there is little reason for postulating separate autocoids for the body and retinal pigments. . . . If the earlier observations of Perkins that the red and yellow pigments are contracted in darkness is correct, the existence of separate hormones would be clearly indicated.[13]

When the responses of the retinal pigments during their persistent cyclic activity are analyzed (Kleinholz, 1937*b*, Table I), no conclusions on the number of hormones concerned in regulating the activity of these effectors can be made. The responses are such that either more than one hormone is involved, or that there is one hormone to which the retinal pigments react independently because of variations in threshold levels. If the latter case is true, then there is no constancy in the relative reactivity of these pigments in different species.

From similar evidence, Brown (1935*a*, 1935*b*) has presented the argument in favor of the multiple hormone hypothesis. The same criticism, namely that the independent responses of the various integumentary chromatophores may be due to different threshold levels to one or two hormones, can be applied to Brown's observations. Brown's explanation has been criticized by Abramowitz (1937*b*), who was led by the results from a study of the comparative physiology of crustacean metachrosis to advocate the unitary hormone hypothesis.

The data presented in the above sections conclusively show the inadequacy of the unitary hormone hypothesis in explaining the behavior of the retinal and integumentary pigmentary effectors. It is clear from the experimental observations that at least two hormones are necessary to reconcile the known facts. The problem as to the actual number of hormones involved in regulating the crustacean pigmentary effectors is far from settled. It can be solved only when these substances have been prepared in pure form and their action on the various components of the pigmentary system tested.

SUMMARY

1. Experiments were performed to determine whether one or more than one hormone is involved in the control of the crustacean pigmentary system.

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[13] Brown (1935*a*) and Perkins (1928) studied *Palaemonetes vulgaris*. Hanström (1937), using the same species, confirmed Perkins' observations of the conditions of the chromatophores in animals kept in darkness.

2. Transfusions of blood from black- and from white-adapted individuals into white-adapted *Crangon* result in the darkening of an equally low percentage of the test animals. Injection of rostral region extract produced darkening in slightly over 50 per cent of the injected animals.

3. Superficial cautery of the rostral region has no permanent effect on color mutability. Deep cautery in 69 animals resulted in 9 individuals which became permanently pale. In each of these 9 *Crangon,* however, swimming and equilibratory movements were abnormal. It is suggested that the deep cautery injured the supraesophageal ganglion, resulting in interference with the regulation of Hanström's sinus gland.

4. The validity of the unitary hormone hypothesis was subjected to a biological test by a study of the retinal and integumentary pigments in *Hippolyte* which has been reported to undergo a diurnal rhythm in the activity of the integumentary effectors.

5. No evidence of persistent activity could be found in the retinal pigments of *H. varians;* the eyes of *H. pleuracantha* showed a persistent cyclic rhythm of the distal retinal pigment, but this was not in the phase expected in *H. varians.*

6. Further study showed that the reported periodicity of color change in *H. varians* was due to a direct effect of darkness on the body chromatophores.

7. The threshold limits of the retinal and integumentary pigment cells to eye-stalk hormones were determined in *Leander adspersus.* The lower threshold limit for the distal retinal pigment was found to be equivalent to 0.016 mg. (wet weight) of eye-stalk; that for the integumentary pigment was 0.0008 mg. These values do not support the unitary hormone hypothesis which requires that the minimal threshold for the retinal pigments be lower than that for the body chromatophores.

8. The responses of the distal retinal pigment to various concentrations of stalk extract are plotted in curves which show a direct relation between the response and the injected dosage.

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REGENERATION AND REORGANIZATION IN UROLEPTUS MOBILIS FOLLOWING INJURY BY INDUCED ELECTRIC CURRENTS

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INTRODUCTION

The interesting investigations of Calkins (1911), Young (1922), and more recently, Dembowska (1925), Taylor (1928), and Reynolds (1932) have demonstrated quite conclusively that regeneration in the Infusoria is accompanied by profound protoplasmic changes. These changes are evidenced by a dedifferentiation and redifferentiation of the regenerating protoplasm comparable to that occurring during binary fission. Thus, it has been shown that the locomotor organelles in the regenerating pieces are entirely absorbed and a complete new set redifferentiated in the reformed organism.

Most investigations along these lines have taken the form of merotomy experiments involving transections, incisions, and excisions of various sorts. This gives rise to the interesting question of whether or not more drastic types of injury or mutilation would result in regeneration of the injured organism. And, if regeneration does occur, is its method of reorganization similar to that observed in other types of mutilations in the ciliates? Relatively few experiments, however, have been performed to test the effects of more drastic mutilations on regeneration in these animals. It is well known that electric currents, under certain intensities, can produce severe injuries to cells and protoplasm in general. The following investigation was designed to determine whether ciliates, injured and mutilated by rapidly induced electric currents, would undergo regeneration and reorganization comparable to that following other reported types of mutilations.

Although careful observations were made on abnormalities in the movements of the organisms (including effects on the motile organelles), vacuolization, and disintegration, attention was particularly centered on the nuclei and on the subsequent behavior of these important cellular constituents.

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[1] Contribution No. 28 from the Department of Biology, Brooklyn College.

Materials and Methods

Uroleptus mobilis was found to be well adapted for studies of this nature, and was used throughout this experiment. An abundant culture was obtained through the kindness of Dr. H. R. Halsey of Columbia University. Sub-cultures were made and maintained in a medium of hay infusion to which was added some " Pablum " and powdered rice. In the procedure described below, only vigorous and flourishing cultures were used, and from these were selected individuals that were not in the process of division. The technique employed throughout was similar to that described in an earlier report (Tittler, 1937). The source of electricity was supplied by two dry-cell batteries in series, attached to an inductorium (Harvard Apparatus Company), which was set for rapidly induced (tetanizing) shocks. A simple key was introduced into the circuit for making and breaking contact. Platinum electrodes from the secondary coil of the inductorium were led into opposite sides of a small " tetanizing " chamber. This latter piece of apparatus consisted simply of an ordinary 1½ by 3-inch glass slide, around the sides of which were built walls of paraffin, arranged in such a fashion as to form a glass-bottomed trough measuring 2 by 4 cm. with a depth of 1.4 cm. The platinum electrodes were fixed at a constant distance of 2.5 cm.

The organisms to be exposed were introduced into the rectangular " tetanizing " chamber, which was then placed on the stage of a binocular microscope. This arrangement permitted free observation of the organisms during and following periods of stimulation. The intensity of the current was controlled by sliding the secondary coil of the inductorium forward or backward as desired. Individual organisms were removed and isolated during or following exposure for future observation. At regular intervals after exposure, *Uroleptus* individuals were fixed on coverslips, stained, and mounted. For permanent preparations, Gilson-Carnoy's and Schaudinn's fluid with acetic acid were employed, followed by Heidenhain's haematoxylin, Mayer's haemalum, and the Feulgen nucleal reaction.

Under the conditions of this experiment, a sixty-second exposure to the current was found to be sufficient to produce various degrees of injury in *Uroleptus.* Currents of greater duration proved lethal, and were not used.

Observations

General

The structure and life history of *Uroleptus mobilis* has been thoroughly presented by Calkins (1919). Consequently, a lengthy discussion of its general morphology at different phases of its life cycle is not warranted. (For a complete critical review, the reader is referred to Calkins, 1933). As Calkins points out, the vegetative macronuclei, usually eight in number, are arranged in a linear fashion within the cell. Occasionally, individuals with nine to twelve macronuclei have been found. The micronuclei are minute and homogeneous and vary in number from two to six. In preparation for division, a typical nuclear cleft appears in each macronucleus, and in each cleft is found a single large granule. This latter structure together with some chromatin is thrown off and the remaining chromatin contents are distributed in the cytoplasm. Subsequently, these " freed " nuclei fuse and condense into a relatively small ellipsoidal and single nucleus. The latter divides two or three times before the cell divides, followed by a fourth division after the daughter cells have separated. The division of the micronucleus is not as complicated. Each divides mitotically and is represented by daughter halves in each of the daughter cells.

Apparently, as in other hypotrichs during binary fission, the motor organelles are resorbed and a new set reformed in each of the resulting individuals.

Under the influence of the induction current the normal movements of *Uroleptus* are considerably affected. The organisms are drawn towards the cathode of the break shocks, at the same time exhibiting a peculiar twisting, lashing movement of the tail region. In some instances a few individuals were seen to swim towards the anode, a phenomenon also observed by Austin (1927). As organisms neared the cathode, marked vacuolization of the cytoplasm occurred, followed by disruption and eventual disintegration of the cell bodies. It was interesting to note that in about 75 per cent of observed cases, as the induced shocks were passed through the culture, the animals were found to be affected and disrupted especially at that end which faced the anode, that is, at the posterior region. This gave rise to many deformed and stumpy animals with the peristomes still intact. During this period pieces of diverse sizes, manifesting various degrees of injury, were obtained. Some of these were isolated for observation. Others were fixed and stained for permanent records.

Figure 1, *A, B, C, D,* shows a few of these individuals fixed immediately after exposure. Different conditions are depicted, ranging from a small stunted fragment, lacking peristome and many macronuclei and micronuclei, to an organism only slightly affected.

Fragments of *Uroleptus,* mutilated by the current, reacted in the following manner. Immediately after removal, anterior pieces with frontal cirri and membranelles still intact, tended to spin in circles for a time, but soon this spinning ceased and the pieces swam in their normal lively manner. Posterior pieces lacking a peristome, remained comparatively motionless for a few seconds and then swam in the customary fashion. These findings confirmed those of Jennings and Jamieson (1902) and others, who demonstrated that pieces of ciliates move in the same essential manner as the entire animal. During this time the wounded edges closed over and began to round out. In less than an

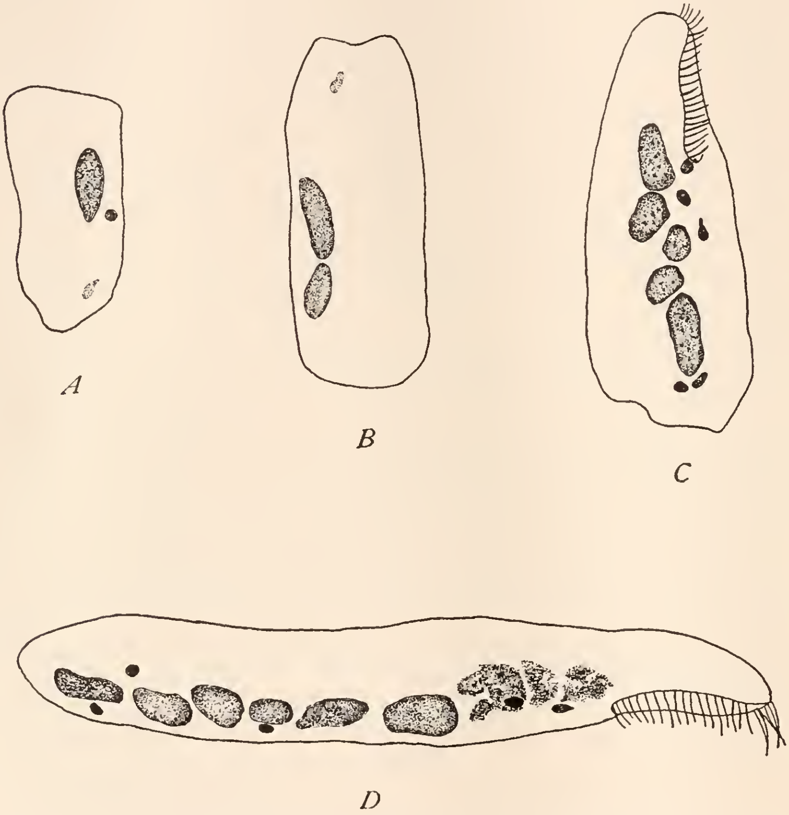

FIG. 1. Camera lucida drawings of material fixed immediately after exposure to the electric current. Cilia omitted.

A. Fragment from posterior region of organism, with one intact macronucleus and two micronuclei. One of the latter is faint and elongate. Peristome lacking. Such fragments do not regenerate. × 1157.

B. Fragment with two macronuclei and one micronucleus, the latter faint and ghost-like. × 1144.

C. Fragment from anterior region of organism. Peristome intact. × 1120.

D. Intact individual. The macronuclei at the anterior region have been disrupted and flattened by the electric current. Remaining macronuclei are unaffected. × 1168.

hour the movements of the pieces slowed down and each was seen to be completely dedifferentiated. Shortly after this it became apparent that a redifferentiation and reorganization was going on within them. It was difficult to observe all the changes which took place in anterior pieces during this protoplasmic reorganization, but as far as could be determined, the cirri and membranelles were withdrawn and a new set developed in the reformed individuals. In posterior pieces, a new peristome appeared, and lost organelles were completely restored. Within three to eight hours each fragment had regenerated its missing structures. At the end of thirty-four hours most of the regenerated individuals were in the process of division. Variations in the time intervals for complete regeneration following injury were probably due to the degree of injury and to whether the regenerating pieces were from anterior or posterior regions of the animal. Thus, it was found that smaller pieces regenerated more slowly than larger ones and often required two days for growth and internal reorganization before dividing. Similarly, regeneration of anterior pieces began earlier than posterior ones.

Nuclei

In material fixed before visible disintegration had occurred, it was evident that the macronuclei in the affected region of the cell were beginning to break up and the nuclear membrane disrupted. The chromatin granules tended to disperse somewhat, resulting in flattened, amorphous nuclei (Fig. 1, *D*). The remaining nuclei were still intact. The micronuclei were elongated and some appeared pale and ghost-like as though they were being absorbed.

In animals stained three to eight hours after exposure the nuclei were found to be in various stages of reorganization. It was evident from a study of fixed stages that this involved the same sort of nuclear reorganization as that which regularly occurs during binary fission in *Uroleptus*. Nuclear clefts appeared and the macronuclei began to fuse with adjacent nuclei and condense into a relatively small division nucleus (Fig. 2, *A, B*). This constricted rapidly and divided into the eight macronuclei characteristic of normal individuals, without the division of the cell (Fig. 2, *C, D, E*). The micronuclei underwent mitotic division, and at times as many as eight of them were found in one cell. However, at the completion of reorganization some of these were absorbed, the number remaining never exceeding six. Occasional individuals were seen to possess ten macronuclei, but the normal nuclear complex was usually restored after normal division. Apparently, regardless of the degree of injury, most fragments possessing two or

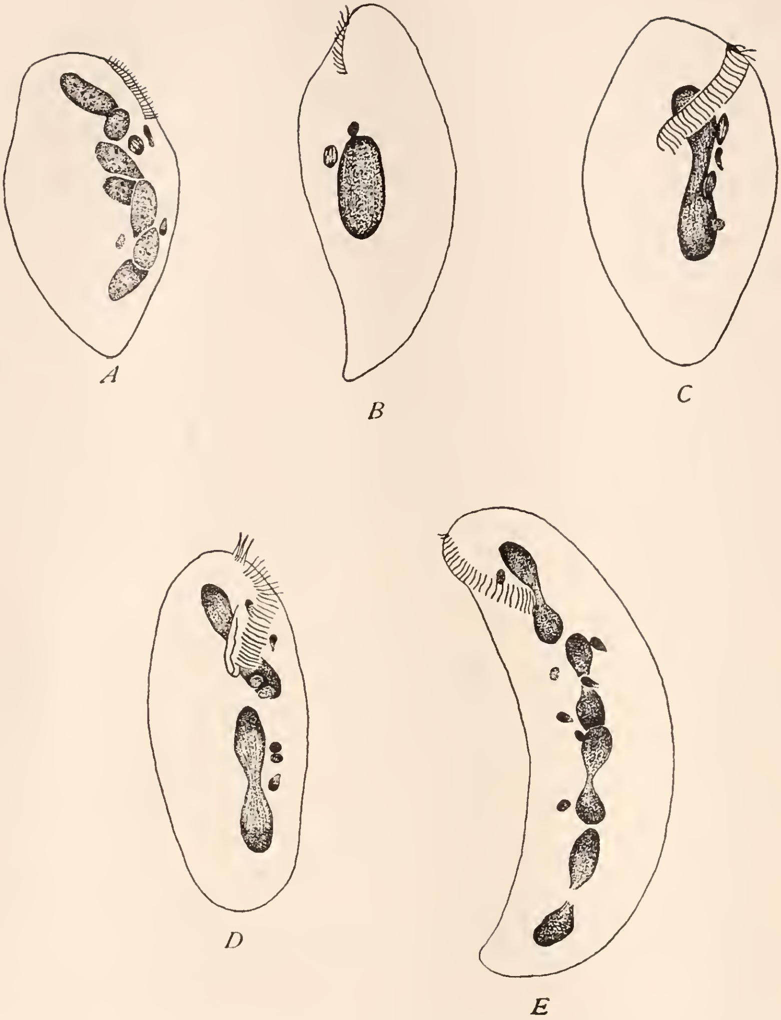

FIG. 2. Camera lucida drawings of individuals fixed three to five hours after exposure to the current. Nuclei in various stages of reorganization. Cilia omitted.

A. Macronuclei beginning to coalesce. One micronucleus in mitosis. × 1052.

B. Later stage showing final fusion of macronuclei into compact division nucleus. × 1087.

C. Division nucleus beginning to constrict into two. Note linear arrangement of chromatin. Two micronuclei in mitosis. × 945.

D. Later stage showing further constriction of the nucleus. × 1001.

E. Individual in late phase of nuclear reorganization, showing final constriction of the nucleus to form eight macronuclei. Division of the cell does not occur during these reorganization changes. × 1013.

more macronuclei and at least one micronucleus were able to reorganize and regenerate. In some instances, smaller pieces, although they contained sufficient nuclear material, were unable to regenerate completely, and perished. Their failure to do so was probably due to an insufficient amount of cytoplasm in which to undergo a reorganization.

In all observed cases of functional regeneration, the stained portions indicated that some macronuclear and micronuclear material had been included in the living fragment. In addition, specimens of these regenerated individuals immediately after division exhibited a perfectly normal nuclear condition.

Discussion

The results here reported on the effects of induced currents on injury and regeneration in *Uroleptus mobilis* bear out the findings of other investigators that mutilations in the hypotrichous ciliates are followed by reorganization changes comparable to those occurring at the time of division. Thus, it has been demonstrated that even after severe degrees of injury and partial cytolysis inflicted by electric currents, *Uroleptus* organisms were able to regenerate and undergo profound reorganization changes involving a dedifferentiation and redifferentiation of the external motor organelles, such as was reported by Calkins (1911) and Young (1922) in *Uronychia,* Dembowska (1925) in *Stylonychia,* Taylor (1928) in *Uronychia uncinata,* Reynolds (1932) in *Oxytricha,* and others.

These results are perhaps more significant because they shed further light on the behavior of the nuclei during regeneration. Balbiani (1891), working with *Stentor,* was probably one of the earliest investigators to point to the interesting fact that the macronucleus goes through precisely the same changes in regeneration as in fission. Johnson (1893) subsequently confirmed this. More recently, Young (1926) found that when *Stylonychia* was transected, the macronucleus and micronucleus in each of the resulting pieces divided into two to restore the normal nuclear complex. However, although later workers were concerned with the respective rôles of the nuclei in regeneration, their interests seemed to lie largely with the problem of the relation of the nuclear content of pieces with regard to functional regeneration—a subject which will be briefly dealt with below. This seems rather surprising in view of the fact that it has become increasingly evident that the behavior of the nuclei in the Infusoria is indicative of deep-seated reorganization changes going on within the cell.

In *Uroleptus* it is apparent that during regeneration the nuclei, particularly the macronuclei, undergo changes which are entirely comparable to those occurring during normal division in this organism (see

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Calkins, 1919). This observation is significant because of its bearing on Taylor's statement (1928) that "There remains, too, the study of internal organelles, such as fibrils and nuclei, which may very possibly undergo changes during regeneration similar to those described above for external motor organs." It would appear, therefore, that in *Uroleptus,* at any rate, the nuclei do undergo such changes during regeneration.

Results obtained from the various merotomy experiments in the Protozoa are in complete agreement with the belief that some nuclear material is necessary for complete regeneration. (For a critical review of the evidence, Reynold's paper (1932) may be consulted.) Gruber's observation (1886) on *Stentor* is the only recorded exception. He found that an enucleated piece from a dividing individual, in which the new peristome had already appeared, was able to develop into a perfect organism, but he failed to give an account of its later history.

Evidently, in most cases, complete functional regeneration seemed to be dependent upon the presence of both macronuclear and micronuclear material. This was found to be true for *Euplotes* (Taylor, 1923, Reynolds, 1932), *Spathidium* and *Blepharisma* (Moore, 1924), and others. Calkins (1911) and Young (1922) were able to show that in *Uronychia* some pieces could regenerate with only macronuclear material at certain periods after the division of the organism, but Young pointed out that these became abnormal, so that for perfect regeneration the micronucleus was necessary. Reynolds (1932), working with an amicronucleate race of *Oxytricha fallax,* found that these organisms were able to regenerate their morphological parts and become normal in every respect. She concluded, however, that the macronucleus of this race was really an amphinucleus.

Uroleptus yielded similar results because, without exception, in all cases where functional regeneration was completed, it was found that some macronuclear and micronuclear material was present in the living fragment. Pieces devoid of such material were able to move in a normal way and even undergo partial regeneration but eventually they died.

SUMMARY

1. *Uroleptus mobilis* organisms were subjected to injury and mutilation by rapidly induced electric currents to determine whether ciliates, drastically injured, would undergo regeneration and reorganization comparable to that following other types of mutilation.

2. Under the influence of the induction current most of the organisms moved abnormally, and were drawn towards the cathode of the break shocks. Disruption and disintegration of the cell bodies occurred,

usually, at the posterior region. This resulted in diverse pieces manifesting various degrees of injury.

3. Injured pieces retained their original polarity and moved in essentially the same manner as the entire animal.

4. Within three to eight hours after injury, each fragment had regenerated its missing structures, and at the end of thirty-four hours most of the regenerated individuals were in the process of division.

5. Regeneration was accompanied by profound protoplasmic reorganization changes comparable to those occurring at the time of division. Cortical organelles were withdrawn, and new sets developed in the reformed individuals. The macronuclei fused into a relatively small division nucleus followed by the rapid constriction of the latter into the eight macronuclei characteristic of normal individuals. The micronuclei divided mitotically.

6. The presence of both macronuclear and micronuclear material was necessary for complete functional regeneration in *Uroleptus*.

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Volume LXXV Number 1

THE
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PUBLISHED BY
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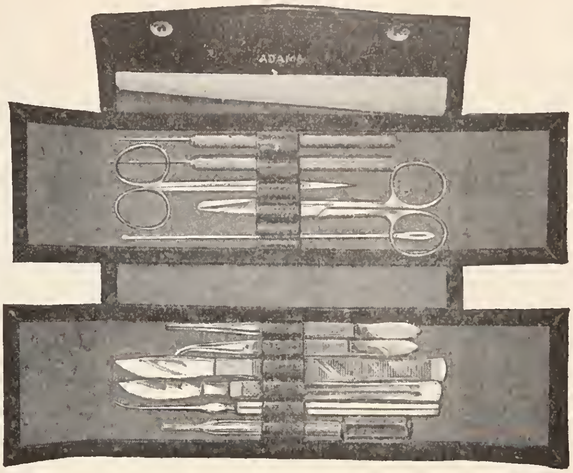

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Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

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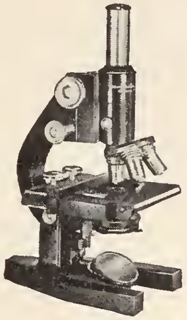

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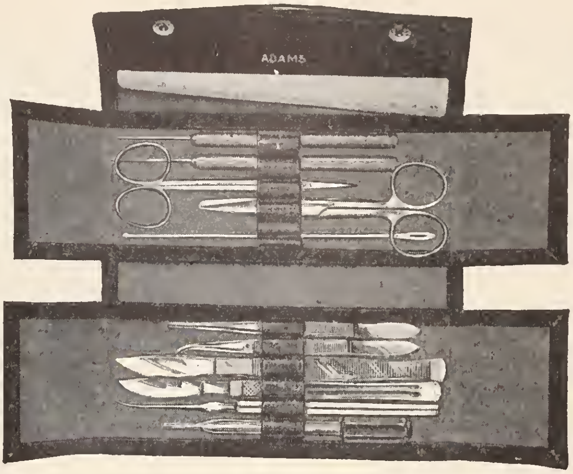

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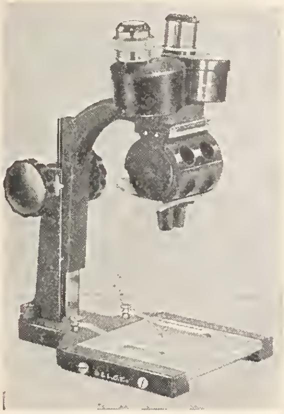

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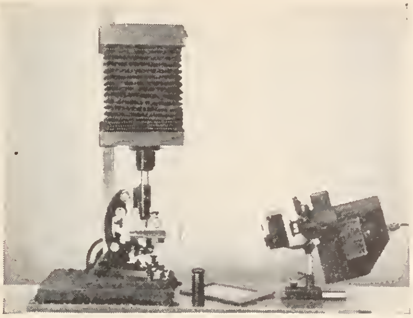

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